Matrix metalloproteinase 8: genetic, diagnostic, and therapeutic approaches

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are hereafter referred to in the text by their Roman numerals (I – IV).

I. Salminen A, Vlachopoulou E, Havulinna AS, Tervahartiala T, Sattler W, Lokki ML, Nieminen MS, Perola M, Salomaa V, Sinisalo J, Meri S, Sorsa T, Pussinen PJ. Genetic variants contributing to circulating matrix metalloproteinase 8 levels and their association with cardiovascular diseases: a genome-wide analysis. *Submitted.*

II. Salminen A, Åström P, Metso J, Solimyani R, Salo T, Jauhiainen M, Pussinen PJ, Sorsa T. Matrix metalloproteinase 8 degrades apolipoprotein A-I and reduces its cholesterol efflux capacity. *FASEB Journal* 2015;29:1435-45.

III. Salminen A, Pussinen PJ, Payne JB, Stoner JA, Jauhiainen M, Golub LM, Lee HS, Thompson DM, Sorsa T. Subantimicrobial dose doxycycline treatment increases serum cholesterol efflux capacity from macrophages. *Inflammation Research* 2013;62:711-20.

IV. Salminen A, Gürsoy UK, Paju S, Hyvärinen K, Mäntylä P, Buhlin K, Könönen E, Nieminen MS, Sorsa T, Sinisalo J, Pussinen PJ. Salivary biomarkers of bacterial burden, inflammatory response, and tissue destruction in periodontitis. *Journal of Clinical Periodontology* 2014;41:442-50.

In addition, some unpublished data are presented.

ABBREVIATIONS

ABC	ATP-binding cassette transporter
ABL	alveolar bone loss
acLDL	acetylated low density lipoprotein
ACS	acute coronary syndrome
AMI	acute myocardial infarction
APMA	4-aminophenylmercuric acetate
apo	apolipoprotein
APR	acute phase response
ATCC	American type culture collection
BOP	bleeding on probing
CAD	coronary artery disease
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CFH	complement factor H
CHD	coronary heart disease
CM	chylomicron
CRP	C-reactive protein
CRS	cumulative risk score
CT	computed tomography
CV	coefficient of variation
CVD	cardiovascular disease
ECG	electrocardiogram
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
eQTL	expression quantitative trait loci
FBS	fetal bovine serum

FC	free cholesterol
FFA	free fatty acid
GCF	gingival crevicular fluid
GE	genomic equivalent
GWAS	genome-wide association study
HBSS	Hank's balanced salt solution
HDL	high density lipoprotein
HuVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule 1
IDL	intermediate density lipoprotein
IFMA	time-resolved immunofluorometric assay
Ig	immunoglobulin
IL	interleukin
IQR	interquartile range
ISD	in source decay
LCAT	lecithin-cholesteryl acyl transferase
LDL	low density lipoprotein
LDLR	LDL receptor
Lp(a)	lipoprotein(a)
LPDS	lipoprotein-deficient serum
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MAF	minor allele frequency
MALDI	matrix-assisted laser desorption/ionization
MI	myocardial infarction
MMP	matrix metalloproteinase
MS	mass spectrometry
OR	odds ratio
oxLDL	oxidized LDL

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PECAM-1	platelet/endothelial cell adhesion molecule 1
PIBI	periodontal inflammatory burden index
PL	phospholipid
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear
PON	paraoxonase
PPD	pocket probing depth
qPCR	quantitative real-time polymerase chain reaction
RCT	reverse cholesterol transport
S100A	S100 calcium-binding protein A
SAA	serum amyloid A
SD	standard deviation
SDD	subantimicrobial-dose doxycycline
SDS	sodium dodecyl sulphate
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SRB1	scavenger receptor B1
TG	triglyceride
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule 1
VLDL	very low density lipoprotein
WHO	World Health Organization
WT	wild type

ABSTRACT

Matrix metalloproteinases (MMPs) are a group of enzymes that are responsible for the degradation of the extracellular matrix (ECM) during e.g. development, repair, and remodeling of tissues. In addition to the ECM components, these enzymes have numerous non-matrix substrates, such as cytokines, growth factors, and signaling molecules. MMP-8, also known as neutrophil collagenase, is released from neutrophils by degranulation when they enter the tissue at the site of inflammation. An imbalance in MMP-8 activity leads to excess degradation of tissues and destructive inflammation, such as periodontitis. MMP-8 is also found in atherosclerotic lesions, where it may contribute to plaque rupture and the development of acute cardiovascular events. Elevated concentrations of MMP-8 in serum and plasma are associated with the risk and outcome of cardiovascular diseases (CVDs). The origin of the elevated MMP-8 levels in CVDs is not entirely clear. Tetracyclines have anti-proteolytic effects in addition to their well-known property of being antimicrobial. Doxycycline inhibits the activity of MMP-8 at both regular, i.e. antimicrobial, and subantimicrobial doses. We investigated the genetic variation that affects circulating MMP-8 levels, the link between MMP-8-associated genetic variants and CVDs, and the role of MMP-8 in lipoprotein metabolism and in diagnostics of periodontitis.

We performed a genome-wide association study (GWAS) in two independent populations with a total of 6049 individuals to identify genetic variants and molecular mechanisms that affect serum MMP-8 concentrations. In addition, we studied whether MMP-8-associated genetic variants are related to increased risk of CVDs and mortality in over 20 000 individuals. According to the GWAS, genetic polymorphism in the gene of complement factor H (CFH) is strongly associated with the concentrations of MMP-8 in serum. By conducting functional experiments with isolated human neutrophils, we found that less MMP-8 was released from neutrophils in response to activation of the alternative pathway of the complement in the carriers of the CFH Ile62 variant compared to the carriers of the Val62 variant. In addition, genetic polymorphism in the locus containing the genes of S100 calcium binding proteins A8, A9, and A12 was associated with serum and plasma MMP-8 levels and also with the prevalence and incidence of CVD in men.

We studied the effect of MMP-8 on the structure and function of apolipoprotein A-I (apoA-I), which is the main protein component of high-density lipoprotein (HDL) particles. In addition, we investigated lipid profiles and the capacity of serum to facilitate cholesterol

efflux from macrophages in an MMP-8 knockout mouse model. We discovered that MMP-8 cleaves apoA-I at its C-terminal end. Pre-treatment of apoA-I and HDL with MMP-8 significantly reduced their ability to promote cholesterol efflux from cholesterol-loaded macrophages. The cleavage of apoA-I by MMP-8 and the reduction in its cholesterol efflux capacity was inhibited dose-dependently by doxycycline at clinically attainable concentrations. MMP-8 deficient mice had significantly lower serum triglyceride levels and larger HDL particle size compared to wild type mice.

We further investigated whether subantimicrobial-dose doxycycline (SDD) treatment affects the ability of serum to promote cholesterol efflux from macrophages in a double-blind, placebo-controlled randomized clinical trial of two years. The subjects treated with SDD had a significant increase in cholesterol efflux from macrophages to the serum compared to the baseline, whereas the efflux levels did not change in the placebo group.

We studied the association of three salivary biomarkers, MMP-8, interleukin-1β, and *Porphyromonas gingivalis*, with periodontal status in 463 subjects with angiographically verified coronary artery disease (CAD) diagnosis. In addition, we evaluated the ability of a novel diagnostic approach, the cumulative risk score (CRS), to detect periodontitis. The concentrations of MMP-8, interleukin-1β, and *P. gingivalis* were associated with the number of deepened periodontal pockets and the extent of alveolar bone loss. The CRS index was more strongly associated with moderate to severe periodontitis than any of the biomarkers alone. The CAD status of the patients did not affect the diagnostic power of the salivary biomarkers.

Our results indicate that the complement system, especially the alternative pathway, contributes significantly to the concentrations of MMP-8 in serum. Genetic polymorphism in *S100A8/A9/A12* locus affects circulating MMP-8 levels, and is associated with CVDs in men. These genetic factors might also be important in other diseases characterized by excess MMP-8 activity. Proteolysis of apoA-I by MMP-8 may disturb HDL metabolism and reverse cholesterol transport, which leads to accumulation of cholesterol in the vessel walls and accelerated atherosclerosis. Inhibition of MMP-8 by doxycycline may reduce the risk of CVDs, especially in vulnerable individuals such as periodontitis patients. Saliva MMP-8, particularly when combined with other biomarkers, has great potential in the diagnostics of periodontitis, regardless of the CAD status of the patients.

1. INTRODUCTION

Matrix metalloproteinases (MMPs) are a group of structurally related enzymes. Their general function is to degrade the extracellular matrix (ECM) during numerous physiological processes, such as development, tissue repair, and tissue remodeling. MMP-8, also known as neutrophil collagenase, is secreted by neutrophils to facilitate their movement in the tissues at the site of inflammation. Neutrophils have a major role in the innate immune system and the clearance of pathogens, and MMP-8 is essential for neutrophil function (Nauseef 2007, Tester et al. 2007). However, neutrophil degranulation and the release and activation of MMP-8 may also cause excessive tissue breakdown and damage, which leads to destructive inflammation. The major substrate of MMP-8 is collagen type I. In addition to collagen and other ECM components, MMP-8 cleaves numerous non-collagenous non-ECM substrates, such as receptors, chemokines, and protease inhibitors, and therefore it may modulate metabolic pathways and the host inflammatory response.

Atherosclerosis is a major cause of cardiovascular diseases (CVDs). The pathogenesis of atherosclerosis is characterized by inflammation and the development of lipid-rich plaques within arterial walls. CVDs are responsible for up to 17.5 million deaths every year, and they are currently the leading cause of mortality and disability in the world (Naghavi et al. 2015). Circulating MMP-8 is a promising biomarker of CVDs. Elevated MMP-8 concentrations in both serum and plasma are associated with the presence of CVD and with the incidence of CVD events (Tuomainen et al. 2007, Momiyana et al. 2010, Tuomainen et al. 2012). Macrophages, endothelial cells, and smooth muscle cells located in atherosclerotic lesions express MMP-8 (Herman et al. 2001). Since MMP-8 degrades collagen and the ECM, it may increase the vulnerability of atherosclerotic lesions and contribute to plaque rupture and the development of acute cardiovascular events. Indeed, elevated concentrations of MMP-8 have been found in unstable atherosclerotic plaques (Molloy et al. 2004). The origin of elevated circulating MMP-8 in CVDs is, however, unclear. Up to 40% of variation in the concentrations of inflammatory biomarkers in the circulation is explained by genetic factors (Schnabel et al. 2009). Therefore, serum MMP-8 concentrations might also be significantly affected by genetic variation.

Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues. It leads to the degradation of soft and hard periodontal tissues, and, eventually to the loss of teeth if not treated. Periodontitis is one of the most common chronic infections in the adult population; in Finland, 64% of adults have signs of the disease, and 21% suffer from advanced periodontitis (Knuuttila and Suominen-Taipale 2008). Periodontitis is initiated by dysbiotic oral microbiota and disturbed host homeostasis (Darveau 2010). Host-derived inflammatory mediators and proteolytic enzymes, such as MMPs, are secreted and activated as a response against the dysbiotic microbiota in the tooth-associated biofilms. Enhanced release and activation of MMP-8 is one of the key factors responsible for tissue degradation during periodontitis. Elevated levels of MMP-8 are found in the gingival tissues, saliva, and the gingival crevicular fluid of periodontitis patients (Sorsa et al. 2016).

Epidemiological and mechanistic evidence indicate that chronic infections, such as periodontitis, are associated with an increased risk for CVDs. The causality between the diseases is, however, not completely clear, and the exact mechanisms linking infections and inflammation to CVDs remain to be clarified.

2. REVIEW OF LITERATURE

2.1 Matrix metalloproteinase 8

2.1.1 General characteristics of MMPs

Matrix metalloproteinases are a group of structurally related enzymes named after the zinc ion at their active site and their ability to degrade ECM. Over 20 MMPs have been identified in humans. The MMPs are classified into six subgroups by substrate specificity and cellular localization: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs. MMPs are able to degrade all components of the ECM. They have an essential role in the development, remodeling, and homeostasis of tissues by regulating functions such as cell migration, differentiation, interaction, and apoptosis (Vu and Werb 2000, Visse and Nagase 2003). In addition to degrading ECM components, MMPs contribute to proteolysis and inflammation by processing bioactive molecules, such as growth factors, receptors, cytokines, and chemokines, and also by activating other MMPs (Vu and Werb 2000, Page-McCaw et al. 2007).

Human MMPs have a conserved domain structure with a pro-domain and a catalytic domain (**Figure 1A**). The pro-domain includes a highly conserved cysteine residue, the so-called "cysteine switch" (Van Wart and Birkedal-Hansen 1990). The cysteine residue interacts with the zinc ion of the catalytic site, keeping the enzyme in catalytically inactive state. When the pro-domain is proteolytically removed or the cysteine thiol group is destabilized, the catalytic site becomes exposed and available for enzymatic activity (**Figure 1B**). The active site at the catalytic domain includes a Zn^{2+} ion bound by three conserved histidine residues (Woessner 1991). Most MMPs also have a hemopexin domain attached to their C-terminal end by a hinge region (**Figure 1A**). The hemopexin domain mediates protein-protein interactions, which contributes to substrate recognition and enzyme activation (Overall 2001).

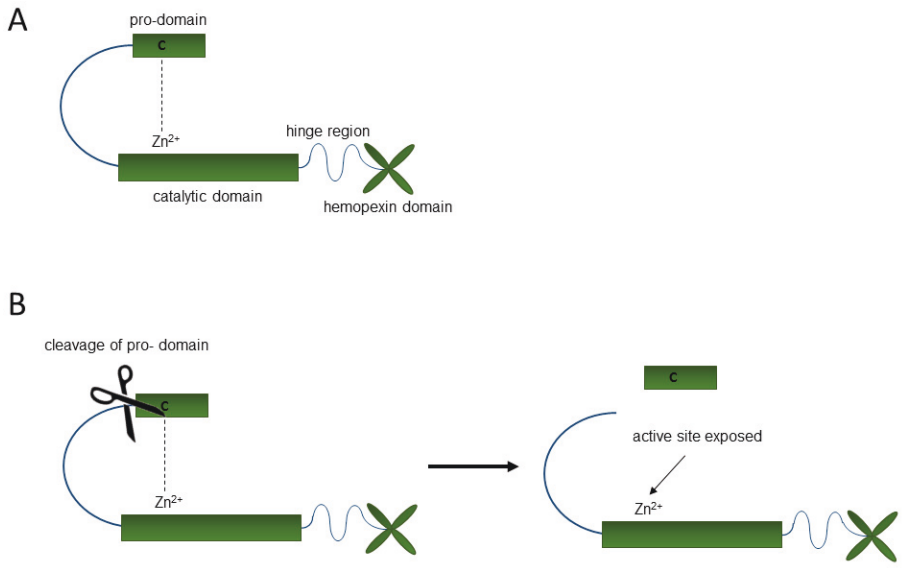

Figure 1. (A) Basic structure of MMPs. The cysteine residue at the pro-domain interacts with the zinc ion at the catalytic site, which keeps the enzyme in a catalytically inactive state. **(B) Activation of MMPs.** When the pro-domain is removed, the active site becomes exposed and available for catalytic activity. Adapted from (Page-McCaw et al. 2007).

The activity of most MMPs is regulated at three main levels: RNA transcription, activation of the proenzyme, and enzyme inhibition. In addition, the activity may be fine-tuned at the levels of protein synthesis, cellular compartmentalization, secretion, cell-surface recruitment, substrate targeting, cellular uptake, and autolysis. (Overall and Lopez-Otin 2002) The transcription and secretion of MMPs are controlled by e.g. inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, growth factors, and hormones (Nagase and Woessner 1999, Overall and Lopez-Otin 2002). The activation of the proenzyme usually requires cleavage of the pro-domain, which results in active enzyme forms that have lower molecular weights. Several proteinases, including endogenous and bacterial proteinases, are capable of activating pro-MMPs. Some MMPs require the action of other MMPs to achieve complete activity. (Tatsuya et al. 2005) MMPs can also be activated by treatment with mercurial compounds, thiol reagents, or reactive oxygen species. These agents probably activate MMPs by disturbing the cysteine-zinc

interaction of the cysteine switch, which results in autoproteolytic cleavage of the pro-domain (Van Wart and Birkedal-Hansen 1990, Bläser et al. 1991).

Under normal physiological conditions, a strict balance between MMP activity and inactivity is maintained. Disruption of the balance may lead to excess tissue destruction and pathological conditions, such as periodontitis or cardiovascular diseases (Pussinen et al. 2013). The most important endogenous inhibitors of MMPs are the specific tissue inhibitors of metalloproteinases (TIMP) -1, -2, -3, and -4. In addition to TIMPs, endogenous non-specific inhibitors, such as α2-macroglobulin and serine proteinase inhibitors, also inhibit MMPs (Herman et al. 2001a). Various synthetic inhibitors have been developed for *in vitro* and therapeutic use. One mechanism of inhibition is the chelation of the Zn^{2+} ion at the active site; the two first inhibitors used in clinical trials, marimastat and batimastat, are mediated by the chelation mechanism (Coussens et al. 2002, Overall and Lopez-Otin 2002). General chelating agents, such as EDTA, can also be used to inhibit MMP activity. Ilomastat (GM6001) is a broad-spectrum MMP inhibitor. It contains a hydroxamic acid group that forms a complex with the zinc at the active site of the MMP, which results in reversible inhibition of MMP activity (Grobelny et al. 1992). Medications such as tetracyclines and bisphosphonates, originally designed for other purposes, also exert inhibitory effects against MMPs (Golub et al. 1998, Teronen et al. 1999).

2.1.2 The structure of MMP-8

MMP-8, also known as neutrophil collagenase or collagenase 2, belongs to the group of collagenases. It has a typical MMP structure that consists of a pro-domain, a catalytic domain, a hinge region, and a hemopexin domain (**Figure 1**). Like most other MMPs, the inactive proenzyme needs to be activated to achieve full catalytic activity. (Van Lint and Libert 2006)

2.1.3 Expression and activity of MMP-8

MMP-8 is mainly expressed by polymorphonuclear (PMN) neutrophils. It is stored as a proenzyme (molecular weight 85 kDa) in the intracellular secondary granules of the neutrophils and released by degranulation in response to inflammatory stimuli (Murphy et

al. 1977). MMP-8 is mainly regulated by factors that affect its release from the neutrophils rather than its *de-novo* biosynthesis. Thus, large amounts of MMP-8 can be immediately released from the cells when needed. Neutrophil degranulation and release of MMP-8 can be induced by e.g. interleukins, TNF-α, and bacterial virulence factors. Following exocytosis, the activation of MMP-8 is mediated by oxidative species released from e.g. the activated neutrophils (Okamoto et al. 1997a), by endogenous proteinases such as cathepsin G, chymotrypsin (Knäuper et al. 1990), and other MMPs (Knäuper et al 1994, Holopainen et al. 2003), and by certain bacterial proteases (Sorsa et al. 1992a, Okamoto et al. 1997b). The active form of PMN-derived MMP-8 has a molecular weight of approx. 65 kDa (Knäuper et al. 1990).

Even though degranulating neutrophils are the main source of MMP-8 in the tissues, MMP-8 is also expressed *de novo* by other cell types. The cells that express MMP-8 include macrophages (Herman et al. 2001b), plasma cells (Wahlgren et al. 2001), epithelial cells (Tervahartiala et al. 2000, Prikk et al. 2001), fibroblasts (Hanemaaijer et al. 1997), smooth muscle cells (Herman et al. 2001b), and endothelial cells (Hanemaaijer et al. 1997). The MMP-8 that is produced by cell types other than the PMN cells is often less glycosylated and has a smaller molecular weight in comparison to PMN-derived MMP-8 (Van Lint and Libert 2006). The same inflammatory mediators that stimulate neutrophil degranulation can induce the expression and synthesis of MMP-8 by non-PMN-lineage cells (Chubinskaya et al. 1996, Hanemaaijer et al. 1997, Abe et al. 2001).

The major substrate of MMP-8 is collagen type I, which is the main structural protein in the ECM of various tissue types. The degradation and remodeling of the vascular basement membrane and ECM by MMP-8 facilitates the transmigration of leukocytes into tissues at the inflammatory site. In addition to ECM proteins, MMP-8 also cleaves numerous non-ECM substrates, such as angiotensin, chemokines, and protease inhibitors (Van Lint and Libert 2006). Substrates of MMP-8 identified by *in vitro* and *in vivo* studies are presented in **Table 1**.

Table 1. Known substrates of MMP-8.

Substrate	Reference
Collagen type I > type III > type II	(Hasty et al. 1987)
Collagen type VII, type X	(Schmid et al. 1986)
Proteinase inhibitor α-1	(Michaelis et al. 1990)
C1 inhibitor	(Knäuper et al. 1991)
Cartilage aggrecan	(Fosang et al. 1993)
Pro-MMP-8	(Knäuper et al. 1993)
Tachykinin substrate P	(Diekmann and Tschesche 1994)
Substance P	(Diekmann and Tschesche 1994)
Bradykinin	(Diekmann and Tschesche 1994)
Angiotensin I and II	(Diekmann and Tschesche 1994, Laxton et al. 2009)
Fibrinogen	(Hiller et al. 2000)
Monocyte chemoattractant protein 1	(McQuibban et al. 2002)
Tissue factor pathway inhibitor	(Cunningham et al. 2002)
Laminin-5	(Pirilä et al. 2003)
LPS-induced CXC chemokine	(Van den Steen et al. 2003)
MIG / CXCL9	(Van Den Steen et al. 2003)
IP-10 / CXCL10	(Van Den Steen et al. 2003)
Estrogen receptor α and β	(Korpi et al. 2008)
Fas ligand	(Korpi et al. 2009)
Macrophage inflammatory protein-1α	(Quintero et al. 2010)
Pro-TNFα	(Lee et al. 2014)

TGF-1β	(Åström et al. 2014)
Human insulin receptor	(Lauhio et al. 2016)

MMP, matrix metalloproteinase; LPS, lipopolysaccharide; MIG, monokine induced by gamma interferon; IP-10, interferon-inducible protein-10; TNF, tumor necrosis factor; TGF, transforming growth factor

MMP-8 is a mediator of both acute and chronic inflammation. Neutrophils have a major role in the innate immunity system and the clearance of pathogens, and the release of MMP-8 is essential for neutrophil chemotaxis and movement in the tissues (Nauseef 2007, Tester et al. 2007). However, neutrophil degranulation and the release and activation of MMP-8 may also cause excessive tissue degradation and damage, which leads to destructive inflammation. Thus, MMP-8 is a central contributor in various inflammatory disorders that are characterized by tissue destruction, such as periodontitis (Sorsa et al. 2004) and rheumatoid arthritis (Sorsa et al. 1992b). It is also involved in cancer progression, wound healing, and numerous other pathological and physiological conditions (Dejonckheere et al. 2011).

2.1.4 The genetics of MMP-8

The gene for MMP-8 is located in the cluster of *MMP* genes in 11q22.3 (Hasty et al. 1990, Pendás et al. 1996). Three single nucleotide polymorphisms (SNPs) in the promoter region of *MMP8*, −799C/T (rs11225395), −381A/G (rs1320632), and +17C/G (rs2155052), affect the activity of the gene (Wang H. et al. 2004). However, the effect of these polymorphisms on the concentrations of MMP-8 in tissues or in the circulation has not been widely investigated. Pradhan-Palikhe et al (2012) studied the association between the *MMP8* SNPs -799C/T and -381A/G and the serum levels of MMP-8 in the patients with arterial disease (n=124) and healthy blood donors (n=100). The -799TT genotype was significantly associated with increased serum MMP-8 concentrations in the control group and whole study population, but not in patients with arterial disease (Pradhan-Palikhe et al. 2012). In another investigation in which 100 systemically healthy individuals were studied, *MMP8* -799 C/T or -381 A/G polymorphisms were not associated with differences in serum MMP-8 concentrations (Aquilante et al. 2007). Genetic variation explains up to 40% of the

variation of the concentrations of circulating biomarkers (Schnabel et al. 2009). Therefore, the concentration of MMP-8 in blood may also be affected by unknown genetic factors.

2.2 Cardiovascular disease and lipoproteins

2.2.1 Lipoproteins

The clinically most relevant lipids in the circulation are triglycerides (TGs) and cholesterol. Triglycerides are a major source of energy and a storage form of lipids in humans. Cholesterol is an essential component of cell membranes, a precursor for certain hormones, vitamins and bile acids, and involved in signaling pathways. Lipids are water-insoluble molecules, and therefore they are combined with amphipathic apolipoproteins for transportation in the circulation. Lipoproteins are complex aggregates of lipids and proteins. Their general function is to transport cholesterol and TGs between organs and tissues for storage, utilization, and degradation. (Brown et al. 1981, Hegele 2009)

Lipoproteins are spherical particles with a hydrophobic core composed mainly of TG and cholesteryl esters (CE), surrounded by an amphiphilic outer layer composed of phospholipids (PL), free cholesterol (FC), and amphipathic apolipoproteins (Steim et al. 1968) (**Figure 2**). Lipoproteins are classified into five main fractions according to their density, protein composition, and function: chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). These main fractions can be divided further into subfractions according to the size, composition, and electrophoretic mobility of the particles. In addition, lipoprotein(a) [Lp(a)] is considered to be a distinct lipoprotein subclass (Krempler et al. 1979). The hydrated density of a lipoprotein particle is proportional to its lipid-protein ratio: the more protein a lipoprotein particle contains, the denser it is. The densest particles have a smaller diameter compared to less dense lipoproteins. Triglycerides are mainly transported by the chylomicrons and VLDL, whereas LDL and HDL are the main carriers of cholesterol. The apolipoprotein composition of lipoproteins varies significantly between the particles. CMs, VLDL, IDL, and LDL contain an apolipoprotein B molecule, which is apoB48 in CM, and apoB100 in VLDL, IDL, and LDL. In addition to apoB, CMs and VLDL also contain apoC, and CMs, VLDL, and IDL contain apoE. HDL particles contain apoA, apoC, and apoE apolipoproteins, but not apoB.

In addition, numerous other types of apolipoproteins have been identified in lipoprotein particles. The composition of particles in lipoprotein fractions is presented in **Table 2**.

Table 2. Composition of human lipoprotein fractions.

Lipoprotein	Density (g/ml)	Size (nm)	FC (%)	CE (%)	TG (%)	PL (%)	Protein (%)	Major apo-lipoprotein
Chylomicron	< 0.95	100-500	1-3	2-4	85-90	3-7	1-2	B-48
VLDL	0.95-1.006	30-80	4-8	12-22	45-65	15-20	6-10	B-100
IDL	1.006-1.019	25-35	8-10	29-35	24-30	19-27	10-20	B-100
LDL	1.019-1.063	18-25	6-10	40-50	5-15	18-24	18-22	B-100
HDL	1.063-1.210	5-12	3-5	13-30	2-10	25-46	40-55	A-I, A-II

VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid. Modified from (Gotto et al. 1986) and (Hegele 2009).

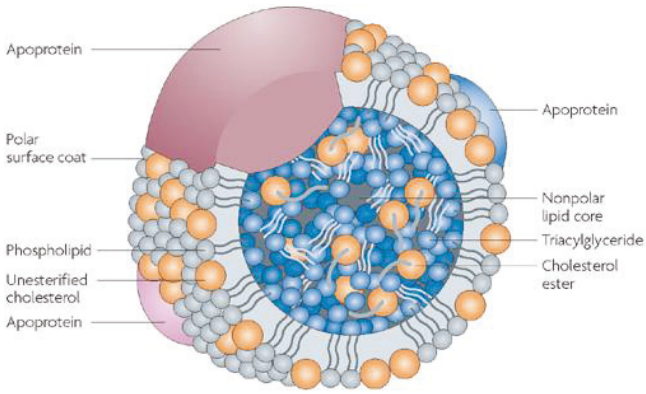

Figure 2. Basic structure of a lipoprotein particle. The hydrophobic core of the particle contains TGs and CEs, whereas PLs, FC, and apolipoproteins are located at the surface of the particle. Reprinted from (Wasan et al. 2008) with the permission of Nature Publishing Group.

2.2.2 The structure of apoA-I and HDL particles

Apolipoprotein A-I is the major protein component of HDL. Most apoA-I in plasma is bound to mature HDL particles. ApoA-I may also be associated with discoidal HDL or exist in lipid-free or lipid-poor configurations. Approximately 5 – 10% of human plasma apoA-I is found in a lipoprotein-unassociated, lipid-free or lipid-poor form (Asztalos 1997, O'Connor 1998). ApoA-I comprises a single polypeptide chain of 243 amino acid residues with an estimated molecular weight of 28 kDa. The peptide chain in organized into eight amphipathic α-helical segments.

The HDL particles are a heterogeneous group that differ in size and composition (Shah et al. 2013). Approximately 70% of the protein content of HDL is composed of apoA-I. In addition, HDL particles may contain other apolipoproteins such as apoA-II, apoC, and apoE, enzymes that are involved in lipid metabolism or have antioxidant activities, such as lecithin-cholesteryl acyl transferase (LCAT) or paraoxonase (PON), and other proteins, such as members of the complement system, proteinase inhibitors, and acute phase response proteins (Shah et al. 2013). ApoA-I is an essential component of HDL; individuals with apoA-I deficiency fail to form normal HDL particles (Matsunaga et al. 1991).

HDL is found in the blood in spherical and discoidal forms. Conventionally, spherical HDL is divided into two classes: larger and less dense HDL_2 and smaller, denser HDL_3. These classes can be further divided into subfractions of HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, and HDL_{3c}. Spherical HDL has a typical lipoprotein structure composed of a hydrophobic core with CE and TG, and an amphipathic surface layer of PL, FC, and apolipoproteins. Discoidal HDL is composed of a bilayer of PL, a small amount of FC, and apoA-I. HDL particles can also be classified according to their mobility in native and two-dimensional gel electrophoresis (Kunitake et al.1985, Kunitake et al. 1992). The majority of HDL has α-mobility, but a small proportion of HDL particles found in plasma displays pre-β mobility.

2.2.3 Overview of lipoprotein metabolism

Lipoproteins are dynamic transporter particles, and their size and composition are constantly modified in the circulation. An overview of endogenous lipoprotein metabolism is presented in **Figure 3**.

Dietary fats are emulsified by bile acids in the intestine and hydrolyzed into free fatty acids (FFAs), monoacylglycerols, and non-esterified cholesterol. After their internalization into the enterocytes, TGs and CE are resynthesized and packaged into CM particles with phospholipids, free cholesterol, and apolipoproteins, particularly apoB48, but also others, such as apoC and apoE (Havel 1997). CMs are secreted into the lymphatic circulation. They enter the subclavian vein via the thoracic duct and are transported to the peripheral tissues. In normolipidaemic individuals, the CMs are recovered in plasma only postprandially. In the endothelial surface of capillaries, lipoprotein lipase (LPL) releases fatty acids from the TGs in CMs. This results in TG-depleted CM remnants, which are transported to the liver where they are taken up by the hepatocytes via LDL receptor (LDLR) or LDL-receptor related protein (Mahley et al. 1989, Hussain et al. 1991). The FFAs released from CMs are transported in the circulation mainly bound to albumin and taken up e.g. by adipose tissue for storage and by muscle cells for energy (Havel 1997).

Only a small amount of circulating cholesterol is derived exogenously from the diet; approximately 80% originates from endogenous biosynthesis. The major endogenous source of lipids is the liver. In the liver, endogenously produced cholesterol and TGs are assembled into VLDL with various apolipoproteins, particularly apoB100, and secreted into the circulation. LPL hydrolyzes TGs in the VLDL into FFAs, which results in VLDL remnant particles. The remnants can be taken up by the liver or remodeled into IDL particles. The IDL particles are hydrolyzed by hepatic lipase and LPL, which yields LDL particles.

LDL particles are the major carriers of cholesterol in the blood (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2002). They are taken up by the cells via LDL receptors by receptor-mediated endocytosis and are then degraded in the lysosomes. Most cells strictly control the intake of cholesterol by regulating the expression and recycling of LDLR. However, macrophages may take in large amounts of cholesterol via scavenger receptors that are not down-regulated by the amounts of intracellular cholesterol, i.e. there is no negative feedback control. This mechanism may lead to the formation of foam cells and the accumulation of cholesterol in, for example, the subendothelial space in arterial walls, and eventually to the development of atherosclerosis. In the liver, which is the main organ responsible for removing LDL from the circulation,

LDL is degraded and the released cholesterol is re-esterified or converted into bile acids for secretion. (Brown et al. 1981)

2.2.4 Reverse cholesterol transport and HDL metabolism

The transportation of cholesterol from peripheral cells and tissues to the liver is mainly mediated by HDL in a process called reverse cholesterol transport (RCT) (Fielding and Fielding 1995).

Lipid-free apoA-I and lipid-poor, disc-like apoA-I particles are synthetized by hepatocytes and intestinal cells. They are also generated from excess surface material in the lipolysis of CM and VLDL particles (Rader 2006). Lipid-poor apoA-I interacts with cells via the ATP-binding cassette transporter A1 (ABCA1) and receives FC and PLs to form nascent, discoidal pre-β HDL (Wang et al. 2001).

Esterification of FC at the surface of nascent HDL by LCAT converts the particles into small, spherical α-HDL (HDL_3). When HDL_3 picks up more cholesterol and PLs, it becomes larger HDL_2. Nascent and small HDL particles receive cholesterol from peripheral cells via ABCA1 (Oram et al. 2000, Du et al. 2015), and mature HDL interacts mainly with ABCG1 and ABCG4 transporters (Wang N. et al. 2004). Along with cholesterol absorption, apoC and apoE are transported to HDL particles from VLDL and IDL.

The CEs of HDL can be transferred to apoB-containing lipoprotein particles, such as LDL, via cholesteryl ester transport protein (CETP), and be replaced by TGs that are transferred in the reverse direction (Stein and Stein 2005). CETP can also exchange CEs for TGs among HDL particle populations. Phospholipids are transferred from tTG-rich lipoproteins to HDL by phospholipid transfer protein (PLTP) (Stein and Stein 2005). In addition, PLTP mediates the conversion of small HDL_3 particles into larger HDL_2 particles and small preβ-HDL particles (Jauhiainen et al. 1993). Thus, the HDL particles are constantly modified in the circulation by the actions of PLTP and CETP. In the liver, HDL is taken up by the hepatocytes via the hepatic scavenger receptor B1 (SRB1) (Acton et al. 1996).

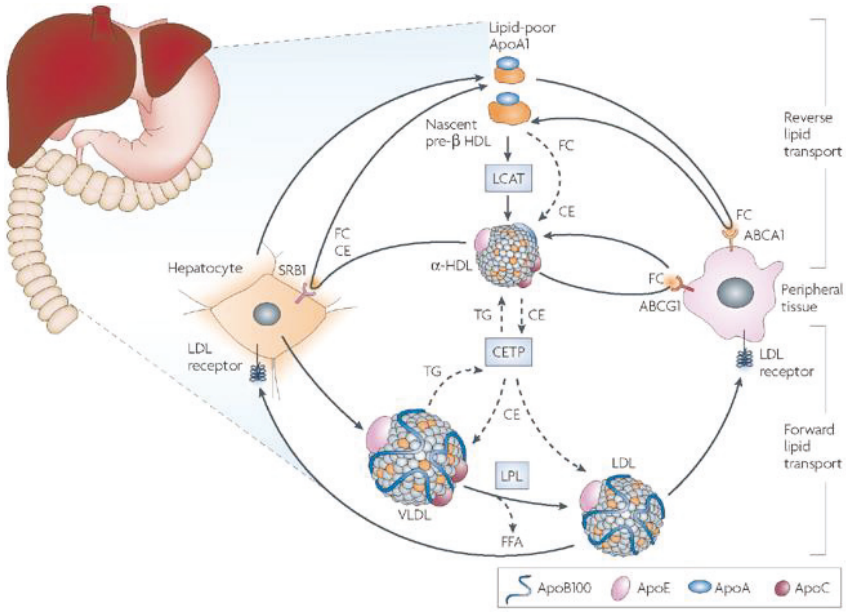

Figure 3. Overview of human lipoprotein metabolism. Reprinted from (Wasan et al. 2008) with the permission of Nature Publishing Group.

2.2.5 Atherosclerosis and cardiovascular disorders

Atherosclerosis is a major cause of cardiovascular diseases. It is a systemic disease characterized by inflammation and the formation of lipid-rich plaques within the arterial walls. Cardiovascular disorders lead to up to 17.5 million deaths every year, and they are the leading cause of mortality globally (Naghavi et al. 2015). Risk factors for CVDs are divided into lipid factors and non-lipid factors, which can be further classified as non-modifiable and modifiable factors. Lipid factors include high levels of LDL or VLDL cholesterol, low levels of HDL cholesterol, elevated serum TGs, and high levels of Lp(a). The modifiable, non-lipid risk factors include smoking, hypertension, diabetes or impaired glucose tolerance, overweight or obesity, physical inactivity, and unhealthy diet. The non-modifiable factors are advanced age, male gender, family history of premature coronary heart disease, and genetic factors. (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2002) Moreover, biomarkers of the disease include C-reactive protein (CRP) measured by the high-sensitivity assay (hsCRP) and serum

homocysteine (Eckel and Cornier 2014). Early signs of atherosclerosis can be found as early as in childhood, but manifestations of the disease are usually encountered in middle-aged or older men and postmenopausal women.

The arterial wall is composed of three layers: the intima, the media, and the adventitia. The innermost layer, the intima, comprises an endothelium, basement membrane, connective tissue, and resident smooth muscle cells (SMCs). The endothelium is a monolayer of specialized epithelial cells that separates the vessel lumen from the subendothelial space. The next layer, the media is a layer of smooth muscle cells that lie in a complex extracellular matrix. The outermost layer, the adventitia, is a dense layer of connective tissue.

The formation of an atherosclerotic lesion is presented in **Figure 4**. Persistently high levels of LDL, IDL, and VLDL in blood leads to their accumulation in the vessel walls (Williams and Tabas 1998). Alteration in the permeability of the endothelium facilitates the infiltration of LDL into the intima where it becomes trapped in the subendothelial matrix (Skalen et al. 2002, Tabas et al. 2007). The endothelium may become irritated and injured as a response to e.g. hypertension, smoking, microbial infection, or haemodynamic forces. The branching sites of arteries are especially prone to atherosclerotic lesions, as the blood flow at these sites is turbulent. In the intima, the lipid and protein components of the trapped LDL become oxidized (oxLDL) and modified by several enzymes, which leads to an inflammatory response (Steinbrecher et al. 1984, Palinski et al. 1989, Parthasarathy et al. 1989, Pentikäinen et al. 2000). Endothelial cells at the site of the lesion express cell adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin, that attract monocytes (Khan et al. 1995, Takei et al. 2001). Monocytes enter the intima, differentiate into macrophages, and take up oxidized and modified lipoproteins by their scavenger receptors (**Figure 4, part a**). When LDL levels in the circulation are persistently high, an imbalance between the influx and efflux of cholesterol leads to the formation of CE-loaded macrophage foam cells in the intima.

The early atherosclerotic lesion is a simple fatty streak. When the lesion evolves, SMCs from the media migrate to the intima (**Figure 4, part b**). They proliferate and synthesize extracellular matrix components, such as collagen and elastin, and form a fibrous cap, a connective tissue layer beneath the endothelial cells. This results in the further accumulation and retention of pro-atherogenic lipoproteins in the intima. The SMCs may also fill up with CE. As excess cholesterol is toxic to cells, the foam cells eventually undergo necrosis and

release their cholesterol content into the extracellular space forming a cholesterol-rich necrotic core of the atherosclerotic lesion (**Figure 4, part c**).

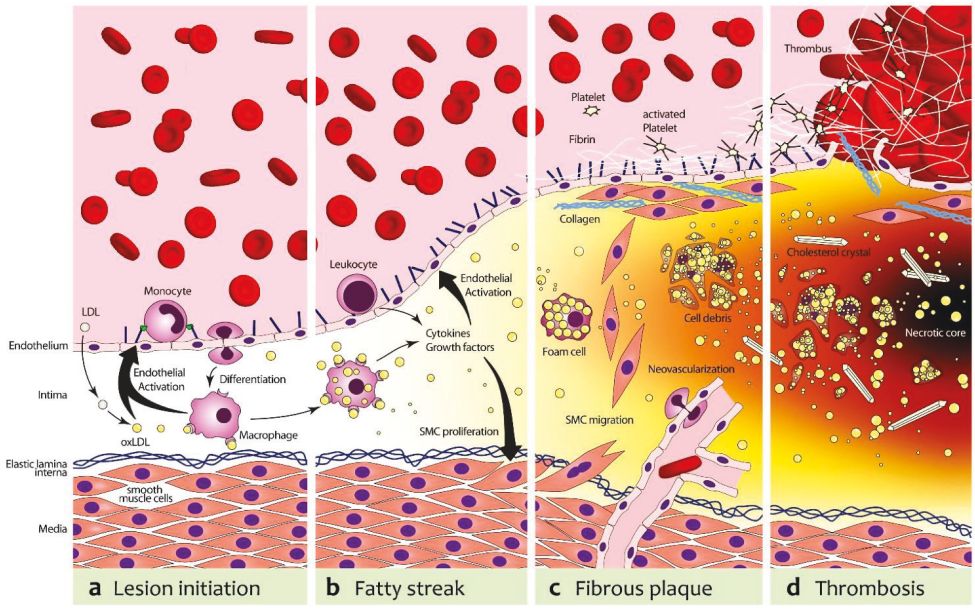

Figure 4. Pathogenesis of atherosclerosis. (a) LDL particles enter the intima and undergo oxidative modification. The oxLDL stimulates the expression of adhesion molecules and chemokines by the endothelial cells, which leads to monocyte recruitment. The monocytes differentiate into macrophages. (b) Macrophages uptake oxLDL and transform into cholesterol-containing foam cells. Cytokines and growth factors stimulate endothelial activation and proliferation of the smooth muscle cells. (c) The increasing volume of the lesion promotes neovascularization. The foam cells eventually undergo necrosis and release cell debris and lipids. The SMCs synthesize ECM components, such as collagen, that forms a fibrous cap beneath the endothelium. (d) Pro-inflammatory and pro-apoptotic processes and physical disruption may result in plaque rupture. Rupture of the plaque leads to thrombosis. Modified from (Steinl and Kaufmann 2015) and reprinted with the permission of MDPI Publishing Services.

The enlargement of atherosclerotic plaques may lead to stenosis of the arteries and tissue ischaemia. Moreover, pro-inflammatory and pro-apoptotic processes and physical disruption may result in plaque rupture and release of intraplaque material into the arterial

lumen. Plaque rupture initiates coagulation of blood and formation of a thrombus (**Figure 4, part d**), which may result in acute events, such as acute coronary syndrome (ACS) or stroke.

Coronary artery disease (CAD), also known as ischaemic heart disease or coronary heart disease (CHD), results from atherosclerosis of the coronary arteries. The formation of atherosclerotic plaques narrows the lumen of the coronary arteries, which results in ischaemia of the heart muscle. A typical symptom of CAD is angina pectoris, but the disease may also remain silent, the first symptom being an acute myocardial infarction (AMI). Diagnostic examinations for CAD include electrocardiogram (ECG), echocardiography, computed tomography scan (CT), and coronary angiography.

2.2.6 Inflammatory background of atherosclerosis

Since the 1990's, inflammation has been identified as a key process in the pathogenesis of atherosclerosis. The inflammatory mechanisms involved in atherosclerosis include both innate and adaptive immunity. Modification of LDL, such as oxidation by myeloperoxidase or reactive oxygen species (Steinbrecher et al. 1984, Palinski et al. 1989, Parthasarathy et al. 1989), or other enzymatic modifications, leads to the expression of chemokines, growth factors, and adhesion molecules by the endothelial cells (Cushing et al. 1990, Rajavashisth et al. 1990, Zernecke et al. 2008). This results in the recruitment of monocytes and other inflammatory cells, such as dendritic cells and T-lymphocytes, to the site of the lesion (Hansson and Hermansson 2011). Monocytes, macrophages, smooth muscle cells, and endothelial cells produce proinflammatory cytokines such as IL-1β, IL-6, and TNFα, and these amplify the inflammatory status within the intima (Libby et al. 2009). In addition, activated mast cells release histamine, proteinases, and heparin (Shi et al. 2015). These events lead to a chronic inflammatory response.

High levels of hsCRP predict future cardiovascular events independent of conventional risk factors (Libby and Ridker 2004). In addition to being a biomarker, CRP itself may contribute to atherosclerosis, e.g. via enhancing endothelial dysfunction (Pasceri et al. 2000, Venugopal et al. 2002). Patients with certain chronic inflammatory disorders have a higher risk of CAD. Such diseases include rheumatoid arthritis, psoriasis, and systemic lupus erythematosus, and several infections, such as periodontitis, *Chlamydia pneumoniae*,

cytomegalovirus, and *Helicobacter pylori* (Mattila et al. 1989, Saikku et al. 1992, Danesh 1999, Franceschi et al. 2009, Grahame-Clarke et al. 2003). Multiple infections cause a pathogen burden that is more strongly associated with CAD than single infections (Epstein et al. 2000).

The acute phase response (APR) leads to changes in lipoprotein metabolism and structure (Sammalkorpi et al. 1988, Khovidhunkit et al. 2004). In general, higher plasma concentrations of VLDL and TGs and lower concentrations of HDL are observed. The size distribution of LDL shifts towards smaller particles, and the lipid composition of VLDL and LDL becomes altered (Khovidhunkit et al. 2000). During APR, HDL undergoes remarkable changes in its protein composition, resulting in proinflammatory particles called acute-phase-HDL (Khovidhunkit et al. 2004). In acute-phase-HDL, the amount of apoA-I decreases and it is replaced by e.g. serum amyloid A (SAA) or lipopolysaccharide-binding protein (Malle et al. 1993, Jahangiri et al. 2009, Jahangiri 2010). The remodeling of HDL particles results in the generation of lipid-poor apoA-I and pre-β HDL (Pussinen et al. 2001, Jahangiri et al. 2009). According to some studies, the capacity of HDL to promote cholesterol efflux is impaired during APR (Jahangiri et al. 2009, McGillicuddy et al. 2009), but there are also contradictory results (van der Westhuyzen et al. 2007, Jahangiri 2010).

2.2.7 Anti-atherogenic mechanisms of HDL

The most recognized anti-atherogenic function of HDL is its ability to promote cholesterol efflux from cells in the arterial walls. In addition to mediating reverse cholesterol transport, HDL has several other atheroprotective features that are unrelated to lipid transportation.

The major protein components of HDL, apoA-I and apoA-II, have antioxidant properties (Garner et al. 1998). Moreover, other proteins that are cotransported with HDL in the circulation, such as PON1, are effective antioxidants that destroy lipid hydroperoxides (Watson et al. 1995). The antioxidative feature inhibits the oxidation of LDL particles in the intima, thereby reducing their atherogenicity. HDL also suppresses the cytokine-induced expression of adhesion molecules by the endothelial cells, which reduces the recruitment of monocytes into the intima (Barter et al. 2004). It also enhances the synthesis of nitric oxide by the endothelium, and can thus ameliorate endothelial dysfunction (Yuhanna et al. 2001, Nofer et al. 2004). Moreover, HDL has anti-coagulant, anti-thrombotic, and antiapoptotic

properties (Epand et al. 1994, Sugatani et al. 1996, Viswambharan et al. 2004, Riwanto et al. 2013).

2.2.8 MMP-8 in atherosclerosis and cardiovascular disease

Several studies suggest that MMP-8 has a role in atherosclerosis. A major component of the fibrous cap in atherosclerotic lesions is collagen type I. Since MMP-8 is a collagenolytic enzyme that efficiently cleaves especially type I collagen, it may increase the vulnerability of atherosclerotic plaques and, thus, contribute to plaque rupture. Indeed, unstable atherosclerotic lesions and progressing atheromas display elevated concentrations of MMP-8 (Molloy et al. 2004, Turu et al. 2006, Peeters et al. 2011), and MMP-8 colocalizes with cleaved collagen type I in the plaques (Herman et al. 2001b). At least macrophages, endothelial cells, and smooth muscle cells express MMP-8 within the atherosclerotic lesions (Herman et al. 2001b).

Animal model studies that use MMP-8 and apoE double knockout mice display atherosclerotic plaques with reduced size, a decreased macrophage infiltration, and an increased collagen content compared to MMP-$8^{+/+}$ $apoE^{-/-}$ mice (Laxton et al. 2009). Fewer endothelial cells, less angiogenesis (Fang et al. 2013), and fewer smooth muscle progenitor cells (Xiao et al. 2013) were observed in the plaques of MMP-8 and apoE double knockout animals. The adhesion of leukocytes onto the vascular epithelium is also reduced in MMP-8 knockouts (Laxton et al. 2009). According to *in vitro* studies, MMP-8 cleaves angiotensin I and generates angiotensin II (Diekmann and Tschesche 1994, Laxton et al. 2009); concordantly, the MMP-8 knockout mice had lower angiotensin II levels and lower blood pressure (Laxton et al. 2009). The MMP-$8^{-/-}$ $apoE^{-/-}$ mice transplanted with MMP-8-deficient SMCs had smaller atherosclerotic lesions than double knockout mice that had received SMCs from wild type mice (Xiao et al. 2013), which suggests that MMP-8 also promotes the migration of SMCs.

In addition to the mouse models, knockdown of endogenous MMP-8 in human umbilical vein endothelial cells (HuVECs) down-regulated the platelet/endothelial cell adhesion molecule-1 (PECAM-1) expression via a reduced conversion of angiotensin I to angiotensin II (Fang et al. 2013). Knockdown of MMP-8 also significantly reduced the migration capacity and proliferation of HuVECs (Fang et al. 2013). A deficiency of MMP-8 in smooth

muscle cell progenitors diminished their ability to migrate through the endothelium and the ECM and into the arterial lesions (Xiao et al. 2014). The activation of MMP-8 in stem cells promoted the development of atherosclerotic lesions (Xiao et al. 2013).

Numerous epidemiological studies suggest that circulating MMP-8 is a promising biomarker of cardiovascular diseases. Elevated serum and plasma MMP-8 concentrations are associated with the presence of CVD as well as with the incidence of CVD events (Tuomainen et al. 2007, Tuomainen et al. 2008, Momiyama et al. 2010, Tuomainen et al. 2012, Pussinen et al. 2013). The exact origin of elevated serum and plasma MMP-8 in CVDs remains unclear.

2.3 Periodontitis

Periodontitis is an inflammatory disease of the tooth-supporting tissues. Its etiology includes environmental (microorganisms of the oral cavity), lifestyle (e.g. poor oral hygiene and smoking), genetic, and systemic factors (e.g. certain systemic diseases). Periodontitis is initiated by disturbances in the dental biofilm and host homeostasis. The host response against the microorganisms leads to local and systemic inflammation, and, eventually, to degradation of the tissues surrounding the teeth. Periodontitis is a widespread disease: according to WHO epidemiological data, its prevalence is 10-15% in adult populations worldwide (Petersen and Ogawa 2012). In the United States, 46% of adults have periodontitis, and 8.9% have the severe form of the disease (Eke et al. 2015), whereas in Finland, 64% of adults display signs of periodontitis (Knuuttila and Suominen-Taipale 2008). Periodontitis, in addition to advanced caries, is a major cause of tooth loss and impaired mastication, and therefore it may have a severe effect on the quality of life. In addition, periodontitis may influence systemic health, as it has been associated with conditions such as CVD, diabetes, and adverse pregnancy outcomes (Mattila et al. 1989, Offenbacher et al. 1996, Grossi and Genco 1998, Sanz and Kornman 2013).

Two major forms of periodontitis are aggressive periodontitis and chronic periodontitis. Additional categories of periodontal diseases include periodontitis as a manifestation of systemic disease, necrotizing ulcerative periodontitis, abscesses of the periodontium, and combined periodontic-endodontic lesions (Armitage 2004). This section mainly focuses on the most common form of the disease, chronic periodontitis.

2.3.1 The structure of the periodontium

The periodontium is the functional unit that is formed by the tissues surrounding and supporting teeth: gingiva, root cementum, periodontal ligament, and the alveolar bone (**Figure 5**). The general function of the periodontium is to support, protect, and nourish the teeth.

The gingiva covers the alveolar bone and the surface of the tooth coronally to the cementoenamel junction. The gingival epithelium is classified into three types: the oral keratinized epithelium, the sulcular parakeratinized epithelium facing the tooth in the gingival sulcus, and the junctional nonkeratinized epithelium attached to the tooth surface. The gingiva, especially the junctional epithelium, provides the first line of defence against oral pathogens. The connective tissue under the epithelium consist mainly of extracellular matrix with type I collagen, and fibroblasts.

The periodontal ligament attaches the root to the alveolar bone. It is formed out of fibrous connective tissue, mainly type I collagen. The cells of the ligament are specialized fibroblasts. The one end of the periodontal ligament is attached to the root cementum and the other end is attached to the alveolar bone. The periodontal ligament absorbs chewing forces, and it contains nerve receptors that provide feedback on the magnitude of those forces to the brain. The periodontal ligament also provides nutrients to the periodontium and participates in the remodeling of the connecting tissue, alveolar bone, and cementum. The cementum is a calcified tissue covering the root apically to the cementoenamel junction. The alveolar bone is the bone that surrounds each tooth.

2.3.2 Pathogenesis of periodontitis

Periodontitis is a complex disease with a multifactorial aetiology. Its pathogenesis involves a dysbiotic microbial state, the host response against the microbes, and the resulting local and systemic inflammation. The severity of periodontal disease ranges from reversible inflammation of the gingiva (gingivitis) to irreversible breakdown of the periodontal tissues, loss of attachment and formation of periodontal pockets, and ultimately the loss of teeth.

Adhesion and colonization of bacteria on the surface of the teeth leads to gingival inflammation that is characterized by swelling, redness, and bleeding. If the inflammation

is restricted to the gingiva, it can be reversed by appropriate treatment. Untreated gingivitis may lead to a more severe inflammation and degradation of deeper periodontal tissues, the periodontal ligament and the alveolar bone. One of the most significant pathological changes in periodontitis is the formation of periodontal pockets. A periodontal pocket is a deepened sulcus between the tooth and periodontal tissues (**Figure 5**, right side). It originates from the migration of the gingival epithelium apically along the surface of the tooth. A periodontal pocket is a favourable site for biofilm accumulation and a niche for anaerobic bacterial species.

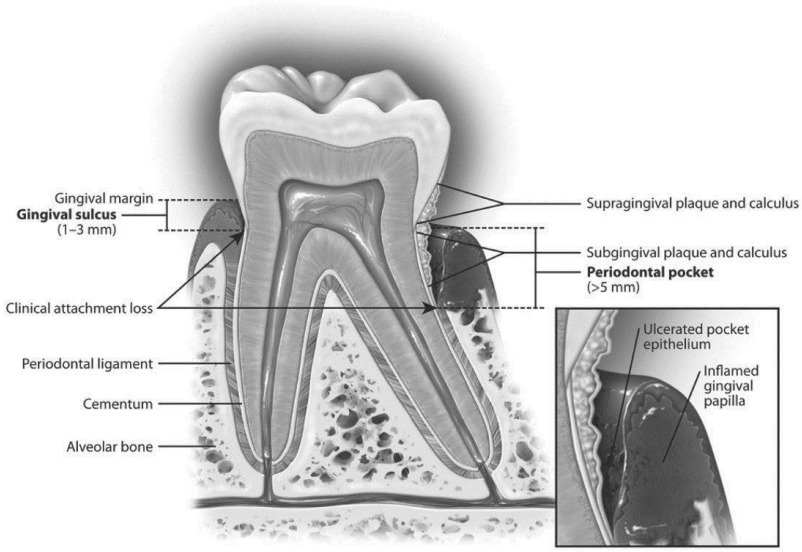

Figure 5. Structure of the periodontium and the effects of periodontitis. The left side of the tooth illustrates a healthy periodontium and the right side represents alterations caused by periodontitis. Reprinted from (Lockhart et al. 2012) with the permission of Wolters Kluwer Health.

Dental biofilm is a complex and dynamic structure in which the bacteria interact with each other. Initial colonizers of the periodontal area adhere to each other by adhesins and form polymicrobial communities. The organisms in the communities are metabolically compatible, and they produce metabolic enzymes that enable the utilization of nutritional substrates more efficiently than for individual species in isolation. The microbes communicate and adapt to the communities, and they may develop collective activity and functions. (Hojo et al. 2009)

In periodontitis, the composition of the subgingival biofilm transforms from the symbiotic community that is characterized by the dominance of Gram-positive bacteria to a dysbiotic state with a majority of Gram-negative bacteria. Certain bacterial species are often found together in the subgingival plaque (Socransky et al. 1998). Of these bacterial complexes, especially the "red complex" which includes *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, is strongly associated with the signs of periodontitis, such as periodontal pocket depth. However, these pathogens do not necessarily initiate the disease *per se*, but the inflammation and periodontal pockets create preferable conditions for the periodontitis-associated pathogens (Wade 2013). The current view is that the microbiota in periodontitis is diverse, and the organisms of the red complex can also be found in the absence of the disease (Könönen et al. 2007, Wade 2013). Plaque volume or the presence of certain pathogen species do not by themselves explain the severity of periodontitis, but the individual susceptibility and host response have a major role. For example, in individuals with genetic susceptibility, even a small amount of biofilm may lead to extensive tissue destruction.

Recent approaches describe periodontitis as a polymicrobial perturbation of host homeostasis (Darveau 2010), and the pathogenesis can be explained by a "polymicrobial synergy and dysbiosis" model (Hajishengallis and Lamont 2012). According to these views, periodontitis is characterized by a synergistic and dysbiotic polymicrobial community rather than by just the presence of bacteria traditionally known as periodontopathogens. The keystone pathogen hypothesis suggests that certain pathogens, such as *P. gingivalis*, may induce the transformation of symbiotic microbial communities of the oral cavity into dysbiotic populations (Hajishengallis et al. 2012). The pathogenesis of periodontitis, including the transition of symbiotic microbiota to dysbiotic microbiota, is schemed in **Figure 6**.

In the healthy state, the host immune response limits bacterial overgrowth in oral microbial communities. A controlled immune-inflammatory state maintains tissue homeostasis, which prevents excessive tissue destruction. Colonization with so-called keystone pathogens such as *P. gingivalis* disturbs the balance between the host and the microbiota, leading to dysbiosis (Hajishengallis and Lamont 2012, Hajishengallis et al. 2012). Even a low concentration of keystone pathogens may significantly modulate the host response and elevate the virulence of bacterial communities by interactive communication with other

bacterial species (Hajishengallis et al. 2012). The manipulation of the host response leads to quantitative and qualitative changes in the composition of biofilms.

The virulence factors of *P. gingivalis* include the capsule, fimbriae, lipopolysaccharide (LPS), lipid A phosphatases, and gingipains. Gingipains are cysteine proteinases. They degrade large peptides and transferrin, which provides nutrients for the bacteria. They are involved in bacterial adhesion and invasion, and in the crosstalk between bacterial species in the biofilm. Gingipains modulate the host response by cleaving antibodies, antimicrobial peptides, complement components, and numerous cytokines, such as ILs and TNF-α (Hajishengallis et al. 2011, Vincents et al. 2011). Together, the virulence factors of *P. gingivalis* subvert the host immune response and increase the pathogenicity of oral microbial communities (Zenobia and Hajishengallis 2015).

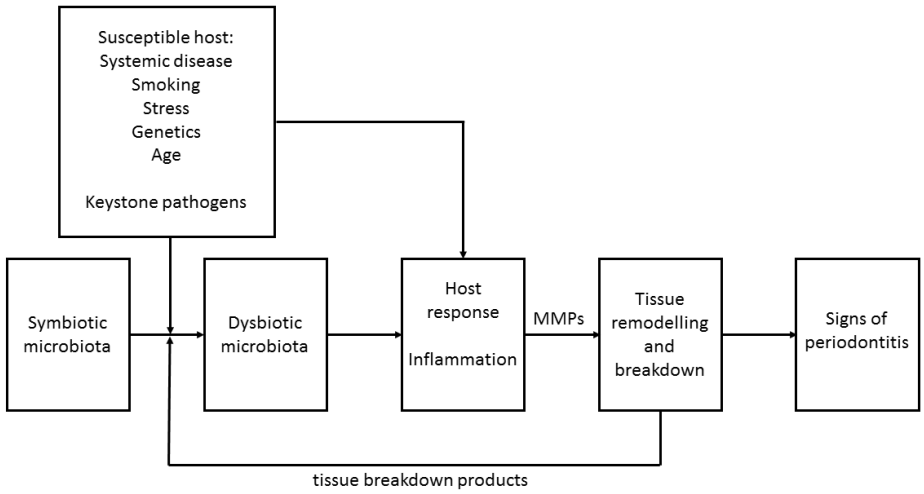

Figure 6. Schematic representation of the pathogenesis of periodontitis. Adapted from (Page and Kornman 1997) and (Hajishengallis 2015).

Periodontitis has an episodic character, with periods of intense host response and periods of resolution of inflammation. Moreover, the disease is not evenly distributed among all teeth, but has sites of predilection. These characteristics further complicate the diagnostics of periodontitis and the evaluation of disease progression.

2.3.3 Host response

Periodontal tissues are in close proximity to the microbes of the dental biofilm. As a defensive mechanism, the tissues express E-selectin, ICAMs, and IL-8 that facilitate the transmigration of neutrophils through the junctional epithelium to the gingival crevice, where they form a wall between the host tissues and the microbes (Tonetti et al. 1998, Darveau 2010). Even in healthy periodontium, approximately 30 000 neutrophils transit through the periodontal tissue every minute (Schiott and Loe 1970, Tonetti et al. 1998). Individuals with congenital or acquired neutrophil deficiencies invariably develop periodontitis (Hart et al. 1994).

In periodontitis, the host immune response is dysregulated and the tissue homeostasis becomes disrupted. The dysregulation of the host response may originate from the challenge caused by the keystone pathogens and the pathogenic microbial state, or from immunoregulatory defects that may be driven by e.g. smoking or diabetes. As a response to dysbiotic microbiota, periodontal cells and inflammatory cells begin to produce cytokines, chemokines, and proteolytic enzymes, which leads to a cascade of inflammation. Neutrophils are considered to be the most important leukocytes that mediate the destruction of periodontal tissues. In the later stages of periodontitis, macrophages, antigen-presenting cells, and lymphocytes are also recruited at the site of inflammation. The expression of e.g. IL-1β, IL-6, and TNF-α by gingival epithelial cells and inflammatory cells amplify the inflammation of the periodontium and lead to the secretion of MMPs by host cells. This results in the destruction of hard and soft periodontal tissues. Thus, host-derived factors are mainly responsible for the tissue destruction in periodontitis. (Darveau 2010)

2.3.4 Risk factors

The established risk factors of periodontitis include poor oral hygiene, smoking, advanced age, male gender, ethnicity, and genetic factors (Genco and Borgnakke 2013). In addition to these, other behavioural and systemic factors, such as alcohol use, unhealthy diet, and stress, have been proposed. Medical conditions that include diabetes, obesity, and osteoporosis may also predispose to periodontal disease. Most of the risk factors, such as smoking, stress, and diabetes, are unlikely to initiate the disease, but rather modify the host immune response (Knight et al. 2016). The genetic factors associated with susceptibility for

periodontitis mainly include polymorphisms in immune response genes (Loos et al. 2005). Genetics is likely to have a stronger role in aggressive periodontitis and in early-onset periodontitis than in chronic periodontitis. The risk factors of periodontitis interact with each other and form a complex interrelationship. For example, heavy alcohol use is associated with poor oral hygiene and low socioeconomic status, and has effects on systemic health and the immune response. Therefore, it is usually impossible to define a single aetiological risk factor or attribute direct causality between risk factors and the disease (Heaton and Dietrich 2012).

2.3.5 Diagnosis and treatment

Usually, periodontitis has only few symptoms. They may include bleeding, redness and swelling of gingiva, halitosis, gingival recession, or movement of the teeth. The clinical diagnosis of periodontitis is based on the measurement of periodontal pockets with a periodontal probe. Usually, a pocket depth of 4 mm or more is considered pathological. In addition to pocket depths, at least bleeding on probing, plaque index, and gingival recessions are recorded. The amount of alveolar bone loss around the teeth is evaluated from radiographs. Loss of bone and attachment reflects the accumulated tissue destruction that has occurred during the current and past episodes of periodontitis.

The basis of the treatment of periodontitis is to eliminate the biofilm and plaque retentions. The subgingival biofilm is removed by scaling and root planing using hand and ultrasonic instruments (Sanz et al. 2012). In addition, local antimicrobial agents, usually chlorhexidine, may be used. Certain severe cases may require systemic antibiotics and surgical therapy (Herrera et al. 2012). Oral hygiene instructions and tobacco counseling are essential for the successful treatment and prevention of periodontitis. Furthermore, all patients need regular supportive periodontal therapy to maintain the treatment outcome (The American Academy of Periodontology 2000).

2.3.6 Biomarkers of periodontitis

The clinical examination of periodontal tissues requires dental professionals and is time-consuming and expensive. For large-scale studies, health promotion, and the screening for

high-risk individuals in the general population, an attractive approach is to measure biomarkers of periodontitis in oral fluids. Biomarkers reflect the current inflammatory activity, whereas bone loss evaluated from radiographs and periodontal pockets might have originated from previous episodes of periodontal tissue destruction (Buduneli and Kinane 2011). Clinical and radiographic examinations may not be optimal for identifying individuals highly susceptible to periodontitis or non-responsive to treatment. This is because subject-level factors, such as microbial composition or the type and magnitude of the immune response have a major effect on the progression of the disease (Offenbacher et al. 2008). Optimally, measurement of biomarkers could aid early detection of periodontitis, the evaluation of response to therapy, and the identification of sites with active disease progression.

Biomarkers of periodontitis include host-derived markers, such as cytokines, MMPs, or byproducts of tissue breakdown, and also pathogen-derived markers such as bacterial DNA. The markers are usually detected in a sample of saliva, mouthrinse, or gingival crevicular fluid (GCF), which are all non-invasively obtained. The GCF is a serum-derived fluid that contains locally produced molecules, such as inflammatory mediators and tissue breakdown products, and systemically derived markers (Golub and Kleinberg 1976). Its flow increases in periodontitis along with increased blood flow and vascular permeability in the periodontal tissues. Samples of GCF are collected from periodontal pockets or gingival sulcus using paper pins. Saliva is a promising diagnostic fluid of periodontitis, since it is easy to collect without specialized equipment or personnel, and it is available in relatively large volumes. Biomarkers in saliva and mouthrinse reflect the overall status of the mouth, whereas the GCF represents an individual site.

As periodontitis is a complex and multifactorial disease, it is unlikely that a single marker of disease activity and prognosis could be found. Up to now, hundreds of studies have evaluated different biomolecules in oral fluids as possible biomarkers of periodontitis (Buduneli and Kinane 2011). Examples of these biomarkers are shown in **Table 3**.

Table 3. Examples of periodontitis biomarkers

Biomarkers of bacterial origin
Bacterial DNA detected by conventional PCR or quantitative PCR
Viable bacteria detected by culture
LPS
Biomarkers of host origin
Inflammatory markers
IL-1β, IL-6, IL-8
TNF-α
Matrix metalloproteinases
MMP-8, MMP-9, MMP-13
Tissue breakdown products
ICTP
Markers of bone remodeling
RANKL, OPG, OPN, cathepsin K
Antibodies against periodontal pathogens
IgG, IgA

LPS, lipopolysaccharide; IL, interleukin; TNF, tumour necrosis factor; MMP, matrix metalloproteinase; ICTP, carboxyterminal telopeptide of type I collagen; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin; OPN, osteopontin; Ig, immunoglobulin

In addition to oral fluids, markers of periodontitis can be detected in samples of serum or plasma (Pussinen et al. 2007a). Periodontitis patients manifest elevated systemic levels of cytokines, CRP, fibrinogen, SAA, and MMPs (Loos et al. 2000, Joshipura et al. 2004, Amabile et al. 2008, Vuletic et al. 2008, Marcaccini et al. 2009). In addition, serum antibody levels against periodontopathogens reflect the host immune response in periodontitis (Pussinen et al. 2011).

2.3.7 MMP-8 in periodontitis

Enhanced expression and activation of host-derived MMPs is a major factor responsible for tissue degradation in periodontitis. MMP-8 is considered as the most important MMP involved in the breakdown of periodontal tissues, because neutrophil-derived MMP-8 is the predominant MMP found in the GCF obtained from periodontal lesions (Sorsa et al. 1988, Sorsa et al. 1990, Sorsa et al. 1994, Ingman et al. 1996, Kiili et al. 2002). MMP-8 in periodontal tissues originates mainly from degranulating neutrophils. In addition to neutrophils, sulcular epithelial cells, gingival and periodontal ligament fibroblasts, and endothelial cells in the periodontal tissues express MMP-8 during periodontitis (Kiili et al. 2002, Sorsa et al. 2006). Proteases produced by periodontal pathogens, such as *P. gingivalis* and *T. denticola*, are capable of activating MMP-8 (Sorsa et al. 1992a). The primary component of the ECM in periodontal tissues is collagen type I, which is the major substrate of MMP-8. In addition to degrading collagen, MMP-8 may influence the immune response in periodontitis by processing non-matrix bioactive molecules, e.g. cytokines such as TGF-β, and chemokines such as CXC chemokines (neutrophil chemoattractants) (Van den Steen et al. 2003, Van Lint and Libert 2006, Tester et al. 2007, Åström et al. 2014).

Because of its central role in the pathogenesis of periodontitis, MMP-8 is among the most studied biomarkers of periodontal disease. Elevated levels of MMP-8 in gingival tissue samples, GCF, mouthrinse, and saliva are associated with the presence and the severity of periodontitis (Sorsa et al. 2016). MMP-8, especially the active form, also reflects the progression of the disease, as well as the response to periodontal treatment (Lee et al. 1995, Chen et al. 2000).

2.4 Periodontitis and cardiovascular disease

The first studies to show a link between oral infections and CVD were published in the 1980's (Mattila et al. 1989, Syrjänen et al. 1989). Since then, numerous epidemiological cross-sectional, case-control, and prospective cohort studies, complemented by several meta-analyses, have found an association between periodontitis and cardiovascular disorders (Bahekar et al. 2007, Humphrey et al. 2008, Blaizot et al. 2009, Orlandi et al. 2014, Leng et al. 2015). The outcomes in these studies have included the presence of CVD, intima-media thickness, carotid plaque thickness, myocardial infarction, acute coronary

syndrome, peripheral arterial disease, abdominal aortic aneurysm, stroke, and cardiovascular death. A recent study on a cohort of nearly 40 000 individuals with more than 15 years of follow-up shows that prevalent and incident periodontitis is associated with an increased risk of CVD (Yu et al. 2015). Additionally, a prospective study with clinical examination and microbiological sampling found that longitudinal improvement in periodontal status and a decreased amount of periodontal bacterial species are related to a decreased progression of carotid atherosclerosis (Desvarieux et al. 2013).

The association between periodontitis and cardiovascular disorders persists even when confounding factors, such as smoking, age, gender, or overweight, are taken into account (Bahekar et al. 2007, Humphrey et al. 2008, Buhlin et al. 2011, Lockhart et al. 2012, Rydén et al. 2016). However, the cause-and-effect relationship between the diseases has been under debate (Lockhart et al. 2012). Periodontitis and CVD share several risk factors, such as smoking, diabetes, stress, and obesity, which are also common in populations. Certain genetic polymorphisms also predispose to both diseases (Schaefer et al. 2009, Kallio et al. 2014). In most observational studies, the association between periodontitis and CVD is partially, but not totally, explained by adjustment for the cardiovascular risk factors (Leng et al. 2015).

Periodontal pathogens, their virulence factors, and endotoxins are able to access the systemic circulation via inflamed periodontal pockets while e.g. brushing or flossing teeth, causing repeated bacteremia (Kinane et al. 2005, Forner et al. 2006, Crasta et al. 2009). In addition, the bacteria may be transported in the circulation inside the phagocytosing cells (Li et al. 2008, Carrion et al. 2012). The bacteria are also able to attach to the surface of leukocytes and erythrocytes via complement receptors (Belstrøm et al. 2011). Periodontal pathogens and their DNA have been found in atherosclerotic plaques in coronary and carotid arteries, in aneurysmal walls, and in intraluminal thrombi (Haraszthy et al. 2000, Ohki et al. 2012, Rangé et al. 2014). Even viable bacteria have been detected in the plaques (Kozarov et al. 2005). However, it is not clear whether the bacteria in the plaques have a role in atherogenesis or whether they have been found by chance. Some periodontopathogens are capable of invading endothelial cells and smooth muscle cells within the plaques (Deshpande et al. 1998, Reyes et al. 2013). The bacteria and their products such as LPS may increase the expression of adhesion molecules and proinflammatory cytokines by the endothelial cells, promoting the adherence of monocytes and the development of local

inflammation in the vascular wall (Doherty et al. 1989, Montgomery et al. 1991). Furthermore, the pathogens and their products may be toxic to the endothelial cells, leading to apoptosis and disturbance in endothelial integrity. *P. gingivalis* and LPS induce LDL aggregation and the transformation of macrophages into foam cells (Giacona et al. 2004, Miyakawa et al. 2004, Hashimoto et al. 2006), and enhance the proliferation of SMCs in the intima (Inaba et al. 2009). Periodontal bacteria are able to activate platelets and induce coagulation, platelet aggregation, and thrombosis (Roth et al. 2006). In addition, they stimulate the production and activation of MMPs by various cell types (Ding et al. 1995).

According to seroepidemiological studies, elevated levels of serum antibodies against periodontal pathogens, especially *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, are associated with prevalent CVD and the risk for stroke, AMI, or future CHD (Pussinen et al. 2003, Pussinen et al. 2004a, Pussinen et al. 2004b, Beck et al. 2005, Pussinen et al. 2005, Pussinen et al. 2007b, Mustapha et al. 2007). A study with a nationally representative sample of almost 7000 individuals in the US found that cardiovascular mortality was lowest in either individuals with low or high levels of antibody against *P. gingivalis* (Sanchez-Torres et al. 2015). Thus, the host response might also have a protective role in the complex relationship between periodontitis and CVD. In addition, individuals with increased serum IgG antibody titres against periodontal bacteria exhibited lowered serum CRP levels and lower serum 8-isoprostane levels, which in turn have an inverse association with CVD (Singer et al. 2015).

Chronic periodontitis leads to systemic inflammation. Locally produced cytokines and inflammatory mediators, such as IL-1β, IL-6, TNF-α, and prostaglandins, are spread into the circulation. These inflammatory mediators may lead to acute phase response, characterized by elevated concentrations of CRP and SAA (Ebersole et al. 2002, Schenkein and Loos 2013). CRP, which is considered a marker of CVDs, is significantly elevated in periodontitis patients (Paraskevas et al. 2008). In addition, cross-reactivity between bacterial and host-derived antigens may contribute to atherogenesis. The systemic antibody response to periodontal pathogens may lead to the production of cross-reactive antibodies against host antigens through molecular mimicry (Schenkein and Loos 2013). The most relevant of such antigens for increased atherosclerosis risk are modified LDL and the members of the heat shock protein family (Buhlin et al. 2015). The gingipain of *P. gingivalis* and chaperonin 60 of *A. actinomycetemcomitans* share molecular identities with epitopes on

modified LDL (Turunen et al. 2012, Wang et al. 2016). Increased oxidative stress is also associated with periodontitis and the pathogenesis of CVDs. Peripheral neutrophils isolated from periodontitis patients exhibited superoxide hyperactivity (Ling et al. 2016).Moreover, the oxidative status is increased both locally in the saliva and GCF, and also systemically, i.e. in the serum of periodontitis patients (Akalın et al. 2007).

Periodontitis is associated with changes in lipoprotein structure and metabolism. Patients with chronic periodontitis have elevated levels of LDL cholesterol and TGs (Lösche et al. 2000, Katz et al. 2002, Joshipura et al. 2004), and also high VLDL cholesterol (Ramirez-Tortosa et al. 2010). In addition, they have low HDL cholesterol and apoA-I levels (Buhlin et al. 2003, Pussinen et al. 2004c, Monteiro et al. 2009). The over-production of reactive oxygen species in periodontitis patients may lead to increased LDL oxidation (Bastos et al. 2012). In addition to oxidation, *P. gingivalis* is able to cleave apoB-100 and modify LDL structure (Miyakawa et al. 2004, Bengtsson et al. 2008). The pro-atherogenic lipoproteins that are isolated from periodontitis patients carry LPS, which leads to macrophage activation and increased uptake of cholesterol (Kallio et al. 2008, Kallio et al. 2013). Periodontitis decreases the cholesterol efflux capacity of HDL and causes changes in HDL metabolism and structure similar to those observed during APR (Pussinen et al. 2004c).

Intervention studies show that periodontal treatment is related to an improvement of atherosclerotic conditions. Improved endothelial function following periodontal intervention has been observed in several studies and meta-analyses (Mercanoglu et al. 2004, Seinost et al. 2005, Elter et al. 2006, Tonetti et al. 2007, Piconi et al. 2009, Gurav 2014, Orlandi et al. 2014). In addition, significantly decreased concentrations of systemic inflammation markers hsCRP, TNFα, and IL-6, decreased concentration of total cholesterol, and increased HDL cholesterol were detected after periodontal therapy in a meta-analysis that included 25 intervention trials (Teeuw et al. 2014). Periodontal patients who already suffered from other additional systemic diseases, such as CVD or diabetes, showed most improvement in response to treatment. However, at present there is insufficient evidence to demonstrate that periodontal treatment *per se* could prevent CVD in periodontitis patients (Li et al. 2014), since randomized controlled clinical trials to evaluate the effect of periodontal interventions on CVD events have not been conducted due to ethical issues.

2.5 Subantimicrobial-dose doxycycline

Tetracyclines are widely used antimicrobial agents. In the 1990's, it was discovered that, in addition to destroying pathogens, tetracyclines also inhibit the degradation of the ECM by downregulating host-derived MMPs (Golub et al. 1995). The MMP-inhibitory property of tetracyclines is unrelated to their antimicrobial activity (Golub et al. 1998).

Doxycycline is the most commonly used tetracycline. It inhibits MMPs by 1) inhibiting the catalytic activity, 2) suppressing gene expression, and 3) preventing the proteolytic and oxidative activation of MMPs (Sorsa et al. 1994, Uitto et al. 1994, Smith et al. 1999). Doxycycline reduces collagenase activity in oral fluids at both regular and low (subantimicrobial) doses (Golub 1995). At the moment, subantimicrobial-dose doxycycline (SDD) is approved as an adjunctive treatment for periodontitis with a dose of 20 mg twice daily. The use of SDD, in adjunction with scaling and root planing, results in statistically and clinically significant gains in clinical attachment levels and reductions in probing depths when compared to those achieved by scaling and root planing alone (Reddy et al. 2003, Preshaw et al. 2004). Doxycycline at subantimicrobial dose targets the host response, not bacteria. It does not have antibacterial effects, and therefore the SDD treatment does not result in the development of resistant strains or the acquisition of multiantibiotic resistance (Preshaw et al. 2004). The frequency of adverse events, such as musculoskeletal syndrome, is also low at these dose levels (Reddy et al. 2003).

In addition to the inhibition of MMPs, doxycycline may also have beneficial effects on systemic inflammation, oxidative stress, and serum lipoproteins. In rats with experimental periodontitis, SDD decreased the total oxidative status and oxidative stress index of serum (Yagan et al. 2014). In a placebo-controlled clinical trial, a 2-year treatment with SDD significantly decreased serum MMP-9 and hsCRP levels in postmenopausal women with periodontitis (Payne et al. 2011). In addition, the treatment increased HDL cholesterol among women who were more than 5 years postmenopausal in the same trial (Payne et al. 2011).

3. AIMS OF THE STUDY

The general aim of this thesis was to investigate genetic variation that affects circulating MMP-8 levels, the link between MMP-8-associated genetic variants and cardiovascular disorders, and the role of MMP-8 in lipoprotein metabolism and in diagnostics of periodontitis.

The specific aims were:

1. To find genetic variants and regulatory mechanisms that affect serum MMP-8 concentration, and to investigate the relevance of these genetic variants in cardiovascular disorders (I).
2. To examine if MMP-8 affects the structure and function of apoA-I and HDL *in vitro* and in an MMP-8 knockout mouse model (II).
3. To study the effect of doxycycline as an MMP inhibitor on the processing of apoA-I by MMP-8 (II).
4. To investigate the effect of SDD treatment on the cholesterol efflux capacity of serum in a placebo-controlled clinical trial (III).
5. To evaluate the potential of salivary MMP-8, IL-1β, *P. gingivalis*, and their cumulative combination in the diagnostics of periodontitis in patients with cardiovascular disorders (IV).

4. STUDY SUBJECTS AND METHODS

A summary of the methods used in the studies I-IV is presented in Table 4. The methods are explained in detail in Section 4.2.

Table 4. Summary of methods used in the studies.

Method	Publication
Genotyping	I
Isolation of human neutrophils	I
Complement activation by inulin	I
Serum and plasma MMP-8 concentration (IFMA)	I
Release of MMP-8 from neutrophils (ELISA)	I
Measurement of complement activity (WIESLAB® immunoassay)	I
Isolation of LDL and HDL	II, III
Preparation of LPDS	II
Preparation of apoA-I-lipid discs	II
Incubation of apoA-I, apoA-I-lipid discs, and HDL with activated MMP-8	II
Protein sequencing by ISD-MALDI-MS	II
Acetylation of LDL	II, III
Radiolabeling of acetylated LDL	II, III
Culturing of THP-1 cells	II, III
Protein concentration (Lowry method)	II, III
Cholesterol efflux from THP-1 cells	II, III
MMP-8 knockout mouse model	II
Serum lipoprotein profiles	II
Serum cholesterol concentration (enzymatic method)	II

Serum triglyceride concentration (enzymatic method)	II
Serum apoA-I concentration (ELISA)	II, III
Serum phospholipid concentration (enzymatic method)	II
Serum PLTP activity	II
Serum PON1 activity	II
SDS PAGE	II
Silver staining	II
Western blotting	II
Serum apoA-II concentration (ELISA)	III
Serum SAA concentration (ELISA)	III
Saliva MMP-8 concentration (IFMA)	IV
Saliva IL-1β concentration (Luminex®-xMAP™ technique)	IV
Saliva *P. gingivalis* quantitation (qPCR)	IV
Statistical analysis	I, II, III, IV

MMP, matrix metalloproteinase; IFMA, time-resolved immunofluorometric assay; ELISA, enzyme-linked immunosorbent assay; LDL, low-density lipoprotein; HDL, high density lipoprotein; LPDS, lipoprotein-deficient serum; apoA-I, apolipoprotein A-I; ISD-MALDI-MS, in source decay matrix-assisted laser desorption/ionization mass spectrometry; PLTP, phospholipid transfer protein; PON1, paraoxonase 1; SDS PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SAA, serum amyloid A; IL, interleukin; qPCR, quantitative polymerase chain reaction

4.1 Study subjects

4.1.1 The Corogene study

The Corogene study is a cohort study that was conducted in the Helsinki University Central Hospital between June 2006 and March 2008 (Vaara et al. 2012). It included 5295 symptomatic patients of Finnish origin that had been assigned to coronary angiogram for any reason. The general purpose of the study was to follow contemporary trends in CHD and its treatment, risk factors, genetics, and epigenetics. The subjects were classified into four groups according to the cardiologic diagnosis and findings of the coronary angiogram:

(1) Patients with no significant CAD with < 50% stenosis of the coronary arteries; (2) Patients with stable CAD with ≥ 50% stenosis in at least one coronary artery; (3) Patients with acute coronary syndrome (ACS) having an episode of chest pain, typical ECG changes, elevated levels of cardiac biomarkers, and ≥ 50% stenosis in at least one coronary artery; and (4) ACS-like, non CAD patients having chest pain with or without changes in ECG, but no significant stenosis in coronary angiogram.

Data on medications, other diseases, previous laboratory measurements, and other relevant information were collected by questionnaires and from hospital records. Patients were considered as having hypertension, dyslipidemia, or diabetes if they were prescribed with medication for these disorders. Patients were defined as current smokers if they had smoked tobacco during the previous six months or had quit smoking within the past six months of the time of examinations. Those who had quit smoking for more than six months prior to examinations were defined as ex-smokers. Blood samples were drawn from the arterial line during angiography.

The study was approved by the Helsinki University Central Hospital Ethics Committee. All patients signed an informed consent form.

4.1.2 The Parogene study

The Parogene study was a sub-study of the Corogene study. The purpose of the Parogene study was to investigate the association between CAD and oral health concentrating on periodontitis (Buhlin et al. 2011). Ten per cent of the Corogene subjects were randomly selected for a complete clinical and radiographic oral examination performed by two periodontists and one radiologist. A total of 506 patients participated in the Parogene study. Stimulated saliva and subgingival plaque samples were collected during the clinical examination and the subjects filled in a questionnaire about their previous dental care and intake of antibiotics.

4.1.3 The FINRISK study

The FINRISK study is a Finnish national population-based survey on non-communicable diseases and their risk factors (Borodulin et al. 2015). The survey has been conducted every

five years since 1972. The study was performed in five geographical areas in Finland in the years 1992, 1997, 2002, and 2007, and it included a clinical examination, questionnaire, and a wide spectrum of laboratory analyses. The study includes up to 20 years of follow-up data.

Cases with prevalent CHD or CVD at baseline were identified 1) as a physician-diagnosed disease in the questionnaire, 2) from the disease-associated drug reimbursement records obtained from the Social Insurance Institution of Finland, and 3) from the National Hospital Discharge register for hospitalizations. The CHD events included AMI, bypass surgery, and coronary angioplasty. The CVD events included CHD events and stroke. The incident CHD events, CVD events, and cause-specific and all-cause deaths during the follow-up were identified via 1) the drug reimbursement records from the Social Insurance Institution of Finland, 2) the National Hospital Discharge register for hospitalizations, and 3) the National Causes-of-Death Register.

The study was approved by the Ethics Committee of the National Public Health Institute (KTL) and all the subjects gave their informed consent.

4.1.4 Postmenopausal osteopenic females with periodontitis

The study on subantimicrobial-dose doxycycline treatment was a placebo-controlled, double-blind, randomized clinical trial that was conducted at the University of Stony Brook, School of Dental Medicine (Stony Brook, NY, USA). The participants were postmenopausal osteopenic females aged between 45 and 70 years with at least nine posterior teeth. They had a history of moderate to advanced chronic periodontitis defined as at least two sites with probing depths of ≥ 5 mm together with bleeding on probing, ≥ 5 mm clinical attachment loss, and radiographic evidence of alveolar bone loss. They were undergoing periodontal maintenance treatment at the moment of the study. The participants had no history of myocardial infarction, stroke, or chest pain. Forty-five subjects who had been randomly assigned to take placebo (n = 26) or doxycycline hyclate (20 mg, n = 19) tablets twice daily for 2 years were included in the study. Blood samples were drawn at baseline, 1-, and 2-year appointments. The Stony Brook Institutional Review Board approved the study protocol and the participants signed an addendum consent form to conduct additional serum analyses.

4.2 Methods

4.2.1 Genotyping and imputation (I)

Genome-wide SNP data on 2500 patients of the Corogene cohort with either ACS or previous myocardial infarction were obtained with the Illumina Human 610K genotyping SNP (Illumina, San Diego, CA) in the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK). Individuals with a non-European background based on multi-dimensional scaling, a failed gender check, low genotyping frequency (< 95%), excess relativeness, or excess heterozygosity were excluded. SNPs were excluded if they had a low call rate (< 95%), a low minor allele frequency (< 1%), or if they were not in Hardy-Weinberg equilibrium ($p < 10^{-6}$). After filtering, the data set was imputed with MACH 1.16 using HapMap 2, release 22 CEU reference population. Imputed SNPs were filtered for high imputation quality ($r^2 > 0.8$) and for minor allele frequency (MAF > 1%), which resulted in ~2.3 million SNPs for the Corogene study subjects.

The FINRISK subjects were genotyped by using Illumina HumanCoreExome, Illumina OmniExpress, Illumina Human 610-Quad, or Affymetrix Genome-Wide Human SNP 6.0 genotyping arrays. Individuals with a failed gender check, excess heterozygosity, or excess relatedness were excluded. The filtered dataset was imputed using IMPUTE v2 and the 1000 Genomes Project EUR population as the reference panel. After imputation, the SNPs were filtered for high imputation quality ($r^2 > 0.8$) and for minor allele frequency (MAF > 1%), which resulted in ~7.4 million SNPs for the FINRISK subjects.

In total, after the exclusions, the genome-wide SNP data and serum MMP-8 concentrations were available for 2203 subjects of the Corogene cohort and 3846 subjects of the FINRISK 1997 cohort.

4.2.2 Measurement of serum and plasma MMP-8 (I) and saliva MMP-8 (IV)

MMP-8 was measured from the serum samples taken from the Corogene subjects and the subjects of the FINRISK 1997 cohort (study I), and from the saliva samples of the Parogene subjects (study IV) with a time-resolved immunofluorometric assay (IFMA) (Medix Biochemica, Kauniainen, Finland) according to the manufacturer's instructions. The interassay coefficient of variation (CV%) was 7.3% and the detection limit 0.08 ng/ml.

After performing the genome-wide association analysis on serum MMP-8, we investigated if the SNPs that were associated with serum MMP-8 were also associated with plasma MMP-8. For this purpose, we randomly chose 100 individuals from the Corogene cohort with each genotype of the SNP rs800292 (GG, GA, and AA; 300 individuals in total), and another 100 individuals with each genotype of the SNP rs1560833 (GG, GA, and AA; 300 individuals in total) who were matched for age, gender, and CAD status. Their citrate plasma samples were analyzed for MMP-8 by the same assay as the serum samples.

We searched for *Trans*- and *Cis*- expression quantitative trait loci (eQTLs) in the Blood eQTL Browser (http://www.genenetwork.nl/bloodeqtlbrowser/) (Westra et al. 2013) to study whether the top SNPs identified from the genome-wide association analysis were associated with gene expression levels.

4.2.3 The Complement activation assay (I)

For the complement activation assay, twelve male subjects with each genotype of the SNP rs800292 (GG, GA, and AA; 36 individuals in total) matched for age and CAD status were selected from the Corogene cohort. The alternative pathway of complement was activated in their serum samples by adding 2 mg/ml inulin. Human neutrophils were isolated from fresh blood of a healthy volunteer by using PolymorphPrep (Axis-Shield PoC, Oslo, Norway) according to the instructions of the manufacturer. Red blood cells were lysed with erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.7) and the neutrophils were placed into Hank's Balanced Salt Solution (HBSS). Inulin-treated serum samples were applied on the neutrophils and the cells were incubated at +37°C for 2 h. The samples were centrifuged at low speed and the supernatants were collected. MMP-8 concentrations were analyzed from the supernatants and sera by Human Quantikine MMP-8 ELISA kits (R&D Systems, Minneapolis, USA) according to the instructions of the manufacturer.The MMP-8 concentrations in the serum samples were subtracted from the MMP-8 concentrations in the supernatants to account for background MMP-8. Mean values of four replicated measurements were used in the statistical analyses.

For evaluation of the activities of complement pathways, one hundred individuals with each of the three genotypes of the GWAS top SNP rs800292 (300 individuals in total) matched for age, gender, and CAD status were randomly chosen from the Corogene cohort. The

activities of classical, alternative, and lectin pathways of complement in serum samples were measured by the WIESLAB® Complement system enzyme immunoassays (EuroDiagnostica, Malmö, Sweden) according to the instructions of the manufacturer. The complement activity levels were expressed as percentage of positive control.

4.2.4 Isolation of human LDL and HDL and bovine LPDS (II, III)

Human LDL, HDL_2, and HDL_3 were isolated from a fresh plasma pool by sequential ultracentrifugation with density cut-offs of 1.019 - 1.063 g/ml for LDL, 1.063 – 1.12 g/ml for HDL_2, and 1.12 – 1.21 g/ml for HDL_3 using solid KBr to adjust the densities.

Lipoprotein-deficient serum (LPDS) was prepared from fetal bovine serum (FBS) by ultracentrifugation using solid KBr to adjust the density, and dialyzed and sterile filtered prior to use.

4.2.5 Preparation of apoA-I-lipid discs (II)

Discoidal proteoliposomes were prepared from apoA-I, phosphatidylcholine (PC), and cholesterol by the sodium cholate dialysis method (Matz and Jonas 1982). The molar ratios of apoA-I:PC:cholesterol in the particles were 1:50:0, 1:50:7, and 1:200:12.

4.2.6 Incubation of apoA-I, apoA-II, apoA-I-lipid discs, and HDL with MMP-8 and inhibitors (II)

Human recombinant MMP-8 (ProteaImmun, Berlin, Germany) was activated by incubating with 1 mM aminophenyl mercuric acetate (APMA). Lipid-free apoA-I, apoA-II, apoA-I-lipid discs with three different compositions, HDL_2, and HDL_3 were incubated with activated MMP-8 in a reaction buffer containing 50 mM Tris-HCl, 200 mM NaCl, and 1 mM $CaCl_2$, pH 7.4. Incubations were performed in the absence and presence of the MMP inhibitors Ilomastat and doxycycline. ApoA-I, apoA-I-lipid discs, and HDL that had been incubated without MMP-8 in the reaction buffer with APMA served as controls.

For SDS PAGE, the reactions were stopped by adding Laemmli sample buffer and boiling for 5 minutes. The contents of the samples were visualized by running 15% SDS PAGE

under reducing conditions and then by silver staining. In addition, Western blotting was performed with a polyclonal and two monoclonal apoA-I antibodies with known epitopes. The epitopes for the monoclonal antibodies were the amino acids 2-8 (N-terminal domain) and 211-220 (C-terminal domain) of human apoA-I.

The cleavage sites of apoA-I were identified by in-source decay matrix-assisted laser ionization mass spectrometry (ISD-MALDI-MS). As a control, the intact apoA-I was also analyzed by ISD-MALDI-MS.

4.2.7 Radiolabeling of LDL (II, III)

Isolated LDL was acetylated in the presence of acetic anhydride (Goldstein et al. 1979) and sterile filtered. Thereafter, acetyl-LDL (acLDL) was radiolabeled by incubating it with [1α,2α(n)^{3}H]cholesteryl oleate (PerkinElmer, Waltham, MA) dissolved in dimethyl sulfoxide.

4.2.8 Cell culture and loading with acetylated LDL (II, III)

Human THP-1 monocytes were obtained from the American Type Culture Collection (ATCC). The cells were maintained in complete RPMI 1640 medium supplemented with 10% (v/v) FBS, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin at +37 °C in a humidified atmosphere of 5% CO_2. To differentiate the cells into macrophages, they were incubated with 100 nM phorbol 12-myristate 13-acetate (PMA) in the growth medium at a density of 500,000 cells/ml for 72 h. To transform the differentiated macrophages into foam cells, they were washed twice with phosphate buffered saline (PBS) and loaded with [1α,2α(n)-^{3}H]cholesteryl oleate–acLDL (10 µg of protein/ml) in RPMI 1640 medium containing 5% LPDS, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin for 48 h. Before the efflux experiments, the cells were washed twice with PBS and the medium was changed to DMEM supplemented with 25 mM HEPES and the antibiotics.

4.2.9 Cholesterol efflux to MMP-8 treated acceptor particles (II)

Lipid free apoA-I, apoA-I-lipid discs, HDL_2, and HDL_3 were incubated with activated MMP-8 in the absence and presence of MMP inhibitors for 12 h. Proteolysis was stopped by 10 mM EDTA and the samples were dialyzed against PBS before the efflux experiments. The acceptor particles were applied to the cholesterol-loaded macrophages (10 µg of apoA-I or 25 µg of HDL protein / ml) and incubated for 16 h. Control cells were incubated in the absence of acceptor particles to measure spontaneous cholesterol efflux to the medium. After incubations, the medium was collected and the cells were lyzed with 0.2 M NaOH. The radioactivity in the medium and the cells were analyzed by liquid scintillation counting. The cholesterol efflux to the medium was calculated as the proportion of cholesterol released into the medium of the total cholesterol in the medium and the cells. Efflux to the incubation medium in the absence of acceptor particles accounting for spontaneous efflux was subtracted.

4.2.10 Cholesterol efflux to patients' serum (III)

Serum (1% v/v) from patients receiving either SDD or placebo was applied to triplicate wells containing acLDL-loaded macrophages and incubated for 16 h. Radioactivity in the medium and the cells was analyzed and the cholesterol efflux to the medium was calculated as in study II.

4.2.11 MMP-8 knockout mice (II)

$MMP8^{-/-}$ mice were a kind gift from Prof. Carlos López-Otín, Oviedo, Spain (Balbín et al. 2003). $MMP8^{-/-}$ mice were backcrossed to the C57BL/6 background. Wild type (WT) C57BL/6 mice were used as controls. Mice (8 $MMP8^{-/-}$ females, 5 $MMP8^{-/-}$ males, 7 WT females, and 8 WT males) were sacrificed at the age of 8 weeks and their fasting blood samples were collected. The experimental protocols were approved by the National Animal Care and Use Committee of Finland.

Serum was analyzed for the concentrations of apoA-I by an ELISA-based assay, and cholesterol and TG levels were determined by enzymatic colorimetric assays. The activity of PLTP was determined by a radiometric assay and the activity of PON1 by a chromogenic

method using paraoxon as a substrate. Serum was depleted of apoB-containing lipoproteins by precipitation with 2 M $MgCl_2$ and 4% sodium phosphotungstic acid (Burstein et al. 1970). Cholesterol efflux from acLDL-loaded THP-1 macrophages to serum (1% v/v) and β-lipoprotein depleted serum (1% v/v) was measured as described in previous paragraphs. Efflux to a control serum was measured on each cell culture plate. The CV% between the plates was 2.2%.

4.2.12 Serum lipoprotein profiles (II)

To obtain lipoprotein profiles of mice, sera from each group of mice ($MMP8^{-/-}$ females, $MMP8^{-/-}$ males, WT females, and WT males) were pooled and aliquots were applied onto a Superose 6HR size exclusion chromatography column previously equilibrated with PBS at a flow rate of 0.5 ml/min. Fractions were collected and analyzed for apoA-I, cholesterol, TG, and phospholipid concentrations.

4.2.13 Measurement of serum apoA-I, apoA-II, and SAA (III)

ApoA-I, apoA-II, and SAA were quantified from the serum samples of subjects receiving SDD or placebo by ELISA-based methods. The concentrations of total cholesterol, HDL cholesterol, TGs, MMP-8, MMP-9, TIMP-1, IL-6, TNF-α, and hsCRP had been analyzed previously (Payne et al. 2011).

4.2.14 Periodontal examination (IV)

The Parogene subjects were examined by two calibrated periodontists and the radiographs were evaluated by a radiologist. At the beginning of the oral examination, the subjects chewed a piece of paraffin for 5 min and at least 2 ml of stimulated whole saliva was collected. Probing pocket depths (PPDs) were measured from six sites of each tooth by using a WHO manual periodontal probe. Bleeding on probing and suppuration were registered from four sites of each tooth. The extent of alveolar bone loss (ABL) was evaluated from panoramic radiographs and graded into four categories: (1) no ABL, (2) mild ABL in the cervical third of the root; (3) moderate ABL in the middle third of the root; and

(4) severe ABL from the apical third of the root to total ABL. In addition to ABL, angular bone defects and periapical lesions were recorded. The periodontal inflammatory burden index (PIBI) (Lindy et al. 2008) was calculated for each subject by adding the number of periodontal pockets with PPD 4–5 mm to the number of periodontal pockets with PPD ≥ 6 mm multiplied by two.

Edentulous subjects were excluded from further analyses. The dentate subjects were divided into two groups based on their periodontal status: 340 subjects with no or mild periodontitis (< 4 sites with PPD of ≥ 4 mm, no ABL or mild ABL) and 123 subjects with moderate to severe periodontitis (patients having at least four sites with PPD of ≥ 4 mm and ABL from moderate to severe).

4.2.15 Measurement of salivary biomarkers (IV)

The concentration of IL-1β in the saliva samples was measured by flow cytometry-based Luminex technology (Milliplex Map Kit; MPXHCYTO-60k, Millipore, Billerica, MA, USA). Quantitative real-time PCR (qPCR) assay for *P. gingivalis* was performed for the saliva samples in an earlier study (Hyvärinen et al. 2012).

4.2.16 Cumulative risk score (IV)

A cumulative risk score (CRS) was calculated for each Parogene study subject by combining the salivary concentrations of MMP-8, IL-1β, and *P. gingivalis* as described by Gursoy et al. 2011. The concentrations of the three biomarkers were divided into tertiles 1–3. Since there was a high number of samples with *P. gingivalis* concentration below the detection limit, the concentration of *P. gingivalis* was divided into tertiles as follows: tertile 1: below the detection limit; tertile 2: above the detection limit but below median concentration (12–23303 GE/ml); tertile 3: above the median concentration (>23303 GE/ml). A cumulative subscore was calculated for each subject by multiplying the corresponding tertile values of MMP-8, IL-1β, and *P. gingivalis* concentrations.Three risk groups were formed based on the cumulative subscores: CRS I (Low risk of having periodontitis): subscores 1 and 2; CRS II (Medium risk): subscores 3, 4, 6, and 8; and CRS III (High risk): subscores 9, 12, 18, and 27.

4.2.17 Statistical analyses (I, II, III, IV)

In study I, associations between serum MMP-8 and genetic polymorphisms were analyzed with linear regression adjusted for age, gender, CAD status (for the Corogene study subjects), a regional indicator based on the five geographical study areas (for FINRISK subjects), and ten first dimensions of the multidimensional scaling on the matrix of the identity-by-state. The genotypes of the SNPs were used as the main explanatory variable and the analyses were conducted for one SNP at a time. For imputed SNPs, estimated allele dosages were used. The dependent variable was log-transformed concentration of serum MMP-8. The analyses were performed with the ProbABEL package, PLINK software, and R software package. The pre-defined threshold for statistical significance was $p < 5*10^{-8}$.

The Corogene and FINRISK 1997 cohorts were first analyzed separately. Subsequently, the results were combined by a fixed-effect meta-analysis based on effect size and standard error using METAL software package (release 2011-03-25, http://csg.sph.umich.edu//abecasis/Metal/) (Willer et al. 2010). The meta-analysis included genomic control correction. The heterogeneity between the samples was investigated with I^2 statistics. Quantile-Quantile (QQ) plots were generated to compare the distribution of observed associations to that expected under the null hypothesis. The genomic control parameters were 1.012 for the Corogene study and 0.999 for the FINRISK 1997 study implying minimal inflation. Conditional regression analysis was performed for the SNPs with strongest associations to identify independent genetic markers.

The associations between plasma MMP-8 concentrations (log-transformed) and the genotypes of rs800292 and rs1560833 were analyzed in subjects matched for age, gender, and CAD status by linear regression assuming an additive model for the minor allele.

The association between the genotypes of rs1560833, rs800292, and rs1061170, and prevalent and incident CHD and CVD events, AMI, stroke, and mortality were analyzed separately in men and women in the FINRISK 1992 (n = 4294), 1997 (n = 5796), 2002 (n = 5974), and 2007 (n = 4549) populations. Logistic regression was adjusted for study cohort, baseline age, and the regional indicator based on the geographical study areas. Time to event analyses were performed by Cox regression adjusted for study cohort and the regional indicator. The time at risk was defined as the age at the time of the event, death, or end of

follow-up. An additive effect for the minor allele of the SNPs was assumed in all models. The analyses were performed by IBM® SPSS® Statistics software (version 22.0).

In study II, the non-parametric Mann-Whitney test was used to analyze the difference between cholesterol efflux levels in the cell experiments and between the apoA-I, cholesterol, and TG concentrations and PON1 and PLTP activities between $MMP8^{-/-}$ mice and WT mice. The threshold for statistical significance was $p < 0.05$. The analyses were performed by IBM® SPSS® Statistics software (version 22.0).

In study III, the distributions of demographic and clinical parameters and medication use were compared between SDD and placebo groups using a 2-sample t-test for continuous variables and the χ^2 test for categorical variables. Changes in the cholesterol efflux capacity of serum relative to the baseline were modeled by linear regression as a function of the study drug using an intent-to-treat analysis with adjustment for the baseline efflux capacity, study visit, and baseline smoking status (a randomization stratification factor) as independent variables. Changes in serum LDL cholesterol, HDL cholesterol, apoA-I, TG, MMP-8, MMP-9, TIMP-1, and hsCRP concentrations were analyzed using a similar model. TG and MMP-8 concentrations were log-transformed as they were not normally distributed. The distribution of 1- and 2-year changes in the outcome variables relative to baseline was compared to 0 using a one-sample t test for within-group analyses.

Pre-specified subgroup analyses were performed based on time since the menopause (within or beyond 5 years) and statin use, using tests of interactions in the regression models. Linear regression models were created to analyze the associations between serum cholesterol efflux capacity (outcome variable) and other serum parameters, which included lipids (cholesterol, TGs), proteolytic enzymes (MMP-8, MMP-9, TIMP-1), inflammation markers (TNF-a, IL-6, hsCRP), and HDL-associated proteins (apoA-I, apoA-II, SAA). The threshold for statistical significance was $p < 0.05$. The analyses were performed with SAS software (SAS Institute Inc., Cary, NC, USA, version 9.1.3).

In study IV, the differences between the characteristics and the periodontal parameters of the study groups (none to mild periodontitis vs. moderate to severe periodontitis) were analyzed by the χ^2 test (categorical variables), the t-test (continuous variables with normal distributions), and the Mann-Whitney test (continuous variables with skewed distributions). Levels of salivary MMP-8, IL-1β, and *P. gingivalis* were expressed as medians with

interquartile range (IQR). The associations of salivary MMP-8, IL-1β, and *P. gingivalis* and the CRS index as tertiles with periodontal parameters and moderate to severe periodontitis were analyzed using a logistic regression model. The models were adjusted for the number of teeth and implants, age, gender, diabetes, CAD status, and smoking. The threshold for statistical significance was $p < 0.05$. The analyses were performed by IBM® SPSS® Statistics software (version 22.0).

5. RESULTS

5.1 Genome-wide association study on serum MMP-8 (I)

5.1.1 Genetic polymorphisms associated with serum MMP-8

The genome-wide association analysis revealed that polymorphisms in two genomic loci were significantly associated with serum MMP-8 concentration (**Figure 7**). Both loci are located in chromosome 1. The statistically most significant association was observed in 1q31.1, which contains the gene for complement factor H (CFH) and CFH-related proteins. The SNP with the strongest association, rs800292, is located in the second exon of *CFH*. It causes a non-synonymous G>A substitution, which results in a change of amino acid Val62>Ile in the CFH protein. The minor allele A of rs800292 was inversely associated with serum MMP-8 ($p = 2.4 * 10^{-35}$). (**Table 5**)

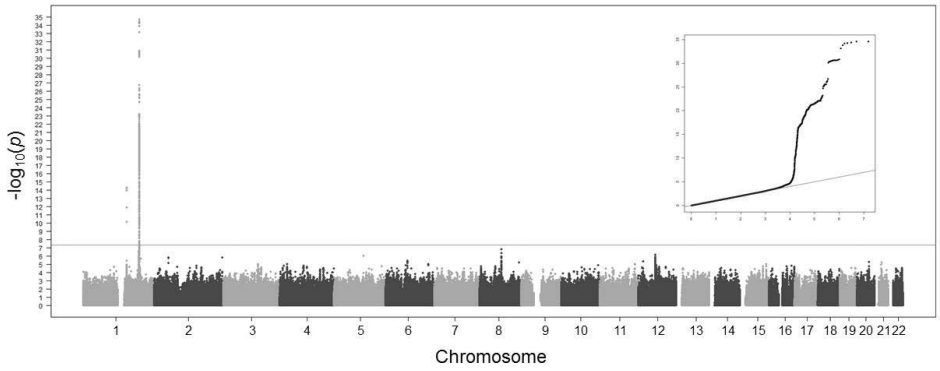

Figure 7. Manhattan plot and QQ-plot of the results from the GWAS of serum MMP-8 concentrations. The results are based on the meta-analysis including 6049 individuals. Two loci with statistically significant associations ($p < 5 * 10^{-8}$) were detected in chromosome 1. The QQ plot indicates small inflation.

The other significant locus was found in 1q21.3, which contains the genes for S100 calcium-binding proteins (S100) A8, A9, and A12. The SNP with the strongest association, rs1560833, is located near the 3′ end of *S100A9*. The minor allele A of rs1560833 was inversely associated with serum MMP-8 ($p = 5.3 * 10^{-15}$) (**Table 5**). Even though rs1560833

is not located in a gene, it is significantly associated with the expressions of *S100A12* (p = 7.6 * 10^{-104}), *S100A8* (p = 1.7 * 10^{-20}), and *S100A9* (p = 6.7 * 10^{-11}) in whole blood according to the eQTL search.

Table 5. Selected SNPs associated with serum MMP-8. Results of the meta-analysis that combined data from the Corogene and the FINRISK 1997 studies.

SNP	Location	Minor allele	MAF	β*	SD	p
rs1560833	1q21.3, downstream of *S100A9*	A	0.28	-0.16	0.02	**$5.31*10^{-15}$**
rs800292	1q31.1, exon of *CFH*	A	0.30	-0.24	0.02	**$2.42*10^{-35}$**
rs1061170	1q31.1, exon of *CFH*	C	0.43	0.16	0.02	**$1.04*10^{-19}$**

* Effect size for log-transformed MMP-8. Models adjusted for age, gender, CAD status (in Corogene), regional factor (in FINRISK), and ten first dimensions of multidimensional scaling. S100A9, S100 calcium binding protein A9; CFH, complement factor H; MAF, minor allele frequency; SD, standard deviation

In addition to serum, rs1560833 was also associated with plasma MMP-8 (p = 0.02), whereas rs800292 was not associated with plasma MMP-8 (**Table 6**). The polymorphisms of *MMP8* promoter (rs11225395, rs1320632, and rs2155052) were not associated with serum MMP-8 concentration.

Table 6. Plasma MMP-8 concentrations in carriers of different rs800292 and rs1560833 genotypes. The concentration of MMP-8 was measured by IFMA.

SNP	Genotype	Plasma MMP-8, ng/ml, median (IQR)	*p**
rs800292	GG	20.64 (29.24)	
	GA	23.89 (36.10)	0.61
	AA	22.11 (28.66)	
rs1560833	GG	19.93 (43.36)	
	GA	18.20 (23.80)	**0.02**
	AA	16.06 (25.97)	

* p-value from linear regression model assuming an additive effect for the minor allele of SNP.
SNP, single nucleotide polymorphism; IQR, interquartile range

5.1.2 Complement activation and MMP-8

The carriage of rs800292 minor allele A (Ile62 variant of CFH) resulted in attenuated release of MMP-8 from neutrophils in response to complement activation ($p = 0.040$) (**Table 7**).

The A allele of rs800292 (Ile62 variant of CFH) was associated with stronger activation of the alternative pathway of complement ($p = 0.002$). No differences were found in the activities of the classical or lectin pathways between rs800292 genotypes. (**Figure 8**)

Table 7. MMP-8 released from neutrophils in response to complement activation. The alternative pathway of the complement was activated in the serum by inulin. The release of MMP-8 from isolated human neutrophils in response to complement activation was measured by ELISA.

		MMP-8 released from neutrophils,ng/ml, mean (SD)	
rs800292	GG	446.0 (71.1)	
	GA	409.4 (50.8)	***p* = 0.040***
	AA	397.3 (42.7)	

* *p* from linear regression assuming an additive effect for the minor allele A. SD, standard deviation

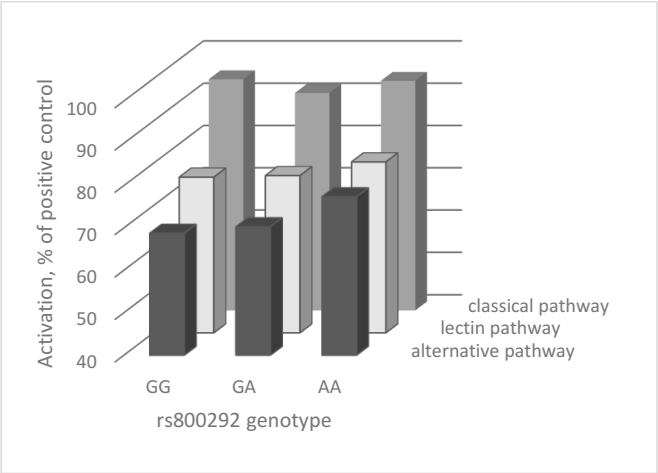

Figure 8. Activation of the complement pathways in serum in the carriers of different genotypes of rs800292. Complement activation was measured in serum samples by the WIESLAB® kit. The only statistically significant difference between the genotypes was seen in the activity of the alternative pathway (black bars).

5.1.3 The association of rs1560833, rs800292, and rs1061170 with cardiovascular disease

Genetic variations of rs800292 or rs1061170 (*CFH*) were not associated with CVDs in FINRISK 1992, 1997, 2002, and 2007 populations. The minor allele of rs1061170 was inversely associated with death ($p = 0.047$) in women. The minor allele of rs1560833 (in the S100 region) was inversely associated with prevalent and incident CVD ($p = 0.032$) and the time of the CVD event ($p = 0.032$) in men, but not in women. (**I, Table 4**)

5.2 The effect of MMP-8 on apoA-I and HDL structure and function (II)

5.2.1 Cleavage of apoA-I and HDL

Incubation of isolated apoA-I, apoA-I-lipid discs, HDL_2, and HDL_3 with activated MMP-8 revealed that MMP-8 cleaves isolated apoA-I and apoA-I within apoA-I-lipid discs. The cleavage occurred in two steps, initially generating a double band of apoA-I, and with higher enzyme concentrations and prolonged incubation time, a single band. Western blotting with monoclonal apoA-I antibodies indicated that the cleavage occurred at the C-terminal end of apoA-I (**II, Figure 2**).

The MALDI-ISD-MS analysis confirmed that MMP-8 cleaves apoA-I from its C-terminal end. The cleavage occurred between amino acids Val^{221}-Leu^{222} and Glu^{191}-Tyr^{192} (**Figure 9**), resulting in two fragments with molecular weights of 25.5 kDa and 22.2 kDa.

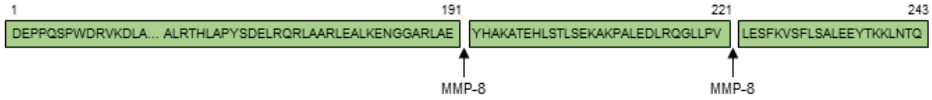

Figure 9. Cleavage of apoA-I by MMP-8. The cleavage sites were determined by MALDI-ISD-MS. The N-terminus of apoA-I remained intact.

ApoA-I-lipid discs with apoA-I:PC:cholesterol molar ratios of 1:50:0 and 1:50:7 were cleaved more efficiently than the discs with the ratio 1:200:12. The cleavage of apoA-I in HDL_2 and HDL_3 by MMP-8 was not detected in SDS PAGE.

5.2.2 Cleavage of apoA-II

Incubation of isolated apoA-II with activated MMP-8 resulted in the cleavage of apoA-II (**Figure 10**). The apoA-II band disappeared also when HDL_2 and HDL_3 were incubated with MMP-8 (**II, Figure 4**).

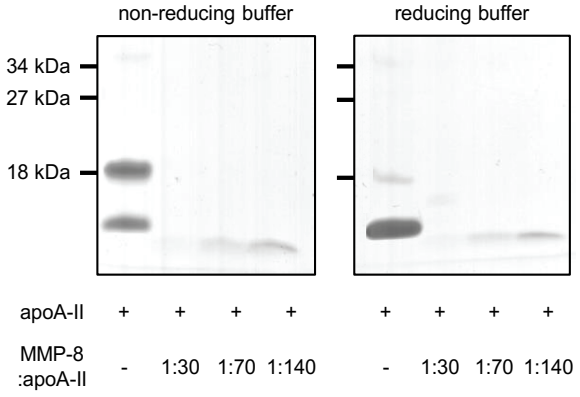

Figure 10. Cleavage of apoA-II by MMP-8. Isolated apoA-II was incubated with activated MMP-8 and the samples were visualized by SDS PAGE followed by silver staining.

5.2.3 The effect of MMP-8 on the cholesterol efflux capacity of apoA-I

Pretreatment of lipid-free apoA-I with various MMP-8 concentrations resulted in a significant reduction in its cholesterol efflux capacity from acLDL-loaded THP-1 macrophages ($p < 0.05$ with all MMP-8 concentrations) (**Figure 11**).

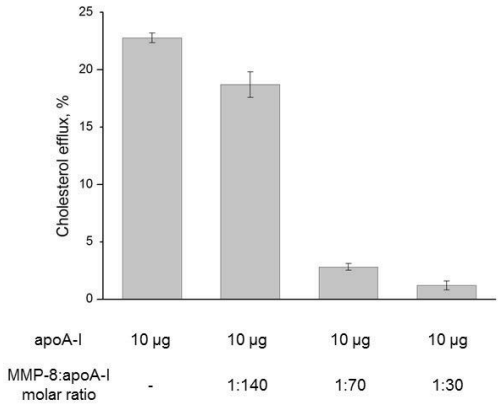

Figure 11. The effect of preincubation with MMP-8 on the cholesterol efflux capacity of apoA-I. The cholesterol efflux capacity of apoA-I from cholesterol-loaded THP-1 macrophages was measured. Three different concentrations of MMP-8 were used in the experiments.

MMP-8 significantly reduced the cholesterol efflux capacities of apoA-I-lipid discs with apoA-I:PC:cholesterol molar ratios of 1:50:0 and 1:50:7. In addition, the cholesterol efflux capacities of HDL_2 and HDL_3 were reduced when treated with MMP-8. However, incubation with MMP-8 did not affect the cholesterol efflux capacity of the discs with apoA-I:PC:cholesterol ratio 1:200:12. (**II, Figure 7**)

5.2.4 The effects of Ilomastat and doxycycline

Doxycycline inhibited the cleavage of apoA-I by MMP-8 in a dose-dependent manner. The cleavage was also partially inhibited by 25 µM Ilomastat. The MMP-8-induced reduction in the cholesterol efflux capacity of apoA-I was also inhibited by doxycycline and Ilomastat (**Figure 12**).

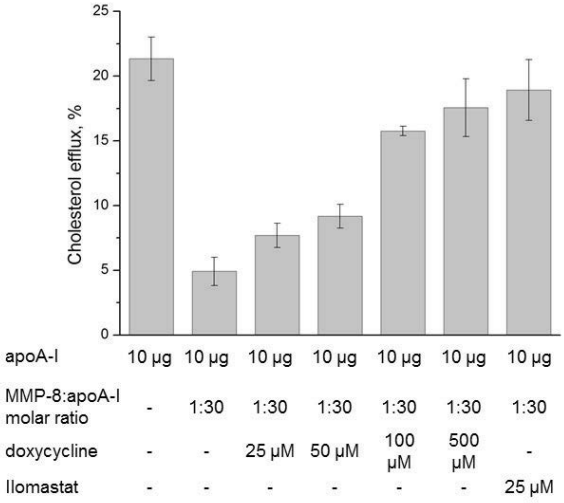

Figure 12. The effect of MMP inhibitors on the cholesterol efflux capacity of MMP-8-treated apoA-I. ApoA-I was incubated with MMP-8 in the presence and absence of doxycycline (at four different concentrations) and Ilomastat. The cholesterol efflux capacity of apoA-I from cholesterol-loaded THP-1 macrophages was measured.

5.2.5 The MMP-8 knockout mouse model

The MMP8$^{-/-}$ mice had lower serum TG concentrations compared to WT mice (p = 0.003 for males and p = 0.002 for females). The serum cholesterol efflux capacities did not differ between the mouse groups. (**Table 8**)

In MMP8$^{-/-}$ mice, the elution positions of major cholesterol and phospholipid peaks in serum fractions were shifted towards larger particle size when compared to controls. In female MMP8$^{-/-}$ mice, also the apoA-I profile shifted towards larger HDL particle size. (**II, Figure 8**)

Table 8. Serum markers of lipid metabolism in MMP-8 deficient and wild-type mice.

	Males			Females		
	WT	**MMP8$^{-/-}$**		**WT**	**MMP8$^{-/-}$**	
	median		*p**	median		*p**
n	8	5		7	8	
body weight, g	22.2	24.3	**0.002**	17.4	18.1	0.13
cholesterol efflux to serum, %	28.4	28.4	1	25.7	23.1	0.05
cholesterol efflux to β-lipoprotein depleted serum, %	26.3	25.1	0.83	24.5	24.4	1
apoA-I, mg/ml	1.87	1.74	0.62	1.32	1.02	0.05
cholesterol, mmol/l	3.07	2.97	0.64	2.30	2.10	0.38
triglycerides, mmol/l	1.12	0.77	**0.003**	0.92	0.66	**0.002**
PON1, µmol/min	89.5	74.0	0.05	96.0	62.5	0.43
PLTP, nmol/ml/h	23145	21705	0.06	23190	21998	0.13

*p-values obtained by the Mann-Whitney test

apoA-I, apolipoprotein A-I; PON1, paraoxonase 1; PLTP, phospholipid transfer protein; WT, wild-type; MMP-8, matrix metalloproteinase 8

5.3 Placebo-controlled randomized clinical trial with subantimicrobial-dose doxycycline (III)

5.3.1 SDD treatment and the cholesterol efflux capacity of serum

There was a significant increase in serum cholesterol efflux capacity in SDD-treated subjects when compared to the baseline at both 1- and 2-year time points ($p < 0.04$ for each) (**Table 9**). There were no significant changes in the cholesterol efflux capacity of serum in the placebo group. Mean cholesterol efflux levels at the first year time point of follow-up were 3.0 percentage points higher among the SDD subjects compared to the placebo subjects (95% CI: 0.7-5.3 percentage points, $p = 0.01$) after adjustment for baseline cholesterol efflux levels and smoking status, while there was no significant difference in 2-year changes (0.7 percentage point increase associated with SDD, 95% CI: 1.8 decrease to 3.1 increase, $p = 0.61$).

Table 9. Changes in serum cholesterol efflux capacity during the 2-year trial.

			Cholesterol efflux (%)	
	Time point	**n**	**Mean (SD)**	***p* ***
Placebo				
	12-month Change from Baseline	26	-0.14 (5.91)	0.90
	24-month Change from Baseline	26	1.64 (4.62)	0.08
SDD				
	12-month Change from Baseline	19	2.83 (5.37)	**0.03**
	24-month Change from Baseline	19	2.66 (4.20)	**0.01**

* comparing mean change to 0 (t-test).

SDD, subantimicrobial-dose doxycycline; SD, standard deviation

5.3.2 SDD and serum concentrations of lipids, lipoproteins, and inflammatory markers

The changes of apoA-I, apoA-II, or SAA levels over the follow-up period did not differ between the SDD and placebo groups. Furthermore, HDL cholesterol, total cholesterol, TG, or MMP-8 concentrations did not differ between the groups. (**III, Table 2**)

Serum apoA-I levels and serum cholesterol efflux capacity were positively associated ($p < 0.001$). In addition, serum cholesterol efflux capacity was significantly associated with total cholesterol, and inversely associated with IL-6 (among placebo subjects). (**III, Table 4**)

5.4 Salivary biomarkers of periodontitis in patients with cardiovascular disorders (IV)

5.4.1 Periodontitis and salivary levels of MMP-8, IL-1β, and *P. gingivalis*

The median concentrations of saliva MMP-8, IL-1β, and *P. gingivalis* were significantly higher in the subjects with moderate to severe periodontitis ($p < 0.001$ for each) when compared to the subjects with no to mild periodontitis.

The median salivary concentrations of MMP-8, IL-1β, and *P. gingivalis* were highest in the subgroups of subjects with severe alveolar bone loss, in subjects with ≥ 7 sites with PPD of 4–5 mm, and in subjects with ≥ 7 sites with PPD ≥ 6 mm. The median salivary concentrations of MMP-8 and IL-1β were highest in subjects with a high percentage of bleeding on probing (BOP). A high salivary concentration of MMP-8 was most strongly associated with BOP and the number of sites with PPD ≥ 6 mm, while a high salivary concentration of IL-1β was most strongly associated with the number of sites with PPD 4–5 mm, the number of sites with PPD ≥ 6 mm, and the PIBI index (**Table 10**). A high salivary level of *P. gingivalis* was most strongly associated with moderate to severe alveolar bone loss (**Table 10**).

Table 10. Odds ratios for the associations between salivary biomarkers and periodontal parameters.

Dependent parameter	**MMP-8**		**IL-1β**		**Pg-qPCR**	
	OR (95% CI)	*p**	OR (95% CI)	*p**	OR (95% CI)	*p**
moderate-total ABL	3.16 (1.72–5.81)	**<0.001**	2.37 (1.30–4.32)	**0.01**	2.99 (1.63–5.51)	**<0.001**
≥ 17 sites with PPD 4-5mm	3.29 (1.84–5.88)	**<0.001**	6.12 (3.21–11.65)	**<0.001**	2.66 (1.44–4.91)	**<0.001**
≥ 7 sites with PPD ≥ 6mm	4.88 (2.23–10.65)	**<0.001**	10.83 (4.02–29.18)	**<0.001**	2.20 (1.10–4.41)	**0.03**
PIBI ≥ 20	3.57 (2.05–6.23)	**<0.001**	6.79 (3.73–12.37)	**<0.001**	2.86 (1.59–5.14)	**<0.001**
BOP ≥ 40%	5.80 (3.20–10.53)	**<0.001**	2.87 (1.66–4.96)	**<0.001**	1.16 (0.66–2.03)	0.60
moderate – severe periodontitis	4.24 (2.26–7.96)	**<0.001**	3.54 (1.89–6.63)	**<0.001**	2.86 (1.57–5.19)	**<0.001**

*Logistic regression adjusted for the number of teeth and implants, age, gender, smoking, diabetes, and CAD status. ABL, alveolar bone loss; PPD, pocket probing depth; PIBI, periodontal inflammatory burden index; BOP, bleeding on probing; OR, odds ratio; IQR, interquartile range

5.4.2 The Cumulative risk score

The CRS index was strongly associated with all periodontal parameters investigated when adjusted for age, gender, smoking, diabetes, CAD status, and the number of teeth. The CRS index had a stronger association with moderate to advanced periodontitis than any of the three salivary biomarkers alone (OR 6.13, 95% CI 3.11 – 12.09, $p < 0.001$). The prevalence of CRS index value III was highest in subjects with a high amount of BOP, several sites with deepened periodontal pockets, and advanced alveolar bone loss (**Table 11**). The CRS index did not correlate with CAD status (**Table 11**).

Table 11. The number of subjects with CRS index I, II, or III in subgroups defined according to CAD diagnosis, smoking or periodontal parameters.

		CRS index		
		I	**II**	**III**
			n (%)	
CAD status	No CAD	35 (34.3%)	39 (38.2%)	28 (27.5%)
	Stable CAD	39 (24.5%)	60 (37.7%)	60 (37.7%)
	ACS	41 (28.3%)	52 (35.9%)	52 (35.9%)
	ACS-like, no CAD	7 (29.2%)	10 (41.7%)	7 (29.2%)
			$p = 0.60$	
Smoking	No	72 (32.4%)	71 (32.0%)	79 (35.6%)
	Ex	46 (24.7%)	71 (38.2%)	69 (37.1%)
	Current	13 (23.6%)	21 (38.2%)	21 (38.2%)
			$p = 0.41$	
BOP tertiles	0 – 25 %	51 (37.0%)	53 (38.4%)	34 (24.6%)
	26 – 44 %	43 (29.5%)	55 (37.7%)	48 (32.9%)
	45 – 100 %	28 (18.9%)	54 (36.5%)	66 (44.6%)
			$p = 0.002$	

Sites with PPD 4-5 mm	0	27 (61.4%)	14 (31.8%)	3 (6.8%)
	1 – 6	49 (40.5%)	49 (40.5%)	23 (19.0%)
	7 – 16	34 (24.6%)	52 (37.7%)	52 (37.7%)
	17 –	12 (9.3%)	47 (36.4%)	70 (54.3%)
			***p* < 0.001**	
Sites with PPD ≥ 6 mm	0	88 (38.3%)	86 (37.4%)	56 (24.3%)
	1 – 3	23 (23.2%)	43 (43.4%)	33 (33.3%)
	4 – 6	6 (15.4%)	17 (43.6%)	16 (41.0%)
	7 –	5 (7.8%)	16 (25.0%)	43 (67.2%)
			***p* < 0.001**	
Alveolar bone loss	none	36 (34.6%)	43 (41.3%)	25 (24.9%)
	mild	61 (31.3%)	74 (37.9%)	60 (30.8%)
	moderate	23 (21.3%)	40 (37.0%)	45 (41.7%)
	severe – total	2 (8.0%)	5 (20.0%)	18 (72.0%)
			***p* < 0.001**	

p-values were obtained by the χ^2 test. CAD, coronary artery disease; BOP, bleeding on probing; PPD, pocket probing depth; CRS, cumulative risk score

6. DISCUSSION

6.1 Genetics of serum MMP-8

6.1.1 The genome-wide association study

Genome-wide association studies are a hypothesis-free method for studying the genetic basis of diseases and quantitative traits, such as the concentrations of biomarkers in blood. The benefit of these studies is that there is no need for selection of candidate genes, and therefore novel and even unexpected variants that contribute to diseases and biological processes can be found. We performed a GWAS on serum MMP-8 concentration in two independent populations. The results revealed that, surprisingly, the genetic variation in the gene of complement factor H is strongly associated with the concentration of serum MMP-8. The SNP with the strongest association, rs800292, causes a Val62Ile substitution in the CFH protein. The frequency of the minor allele of rs800292 was 30%, making it a relatively common variant. Interestingly, genetic polymorphism in the locus that contains genes for S100 calcium binding proteins A8, A9, and A12 is also associated with serum MMP-8 levels.

6.1.2 Complement activation and the effect of sample preparation

Our results show that the Ile62 variant of CFH is associated with a lower MMP-8 concentration in serum. CFH is an important inhibitory regulator of the alternative pathway of complement. According to a previous study, the CFH Ile62 variant increases the binding of CFH to C3b, which therefore leads to decreased complement activation compared to the Val62 variant (Tortajada et al. 2009). The carriers of the CFH Ile62 variant also have lower serum levels of C3a desArg, indicating less complement activation compared to the carriers of the Val62 variant (Reiner et al. 2013). We hypothesized that the attenuation of complement activity that results from the Ile62 variation of CFH leads to decreased neutrophil activation and degranulation, and thereby decreases the release of MMP-8 from neutrophils in response to complement activation. Indeed, in functional experiments with isolated human neutrophils, we found that less MMP-8 was released from neutrophils in

response to the activation of the alternative pathway of complement in the carriers of the CFH Ile62 variant.

We also found that genetic variation of *CFH* did not have an effect on MMP-8 concentration in plasma. Therefore, the activation of complement might occur during the preparation of serum samples, which results in degranulation of neutrophils. The association between CFH polymorphism and MMP-8 concentration was not observed in the plasma samples, probably because anticoagulants used in the preparation of plasma inhibit the complement activation (Mollnes et al. 1988). It is also possible that anticoagulants in plasma samples enhance the degradation of MMPs in the absence of calcium (Makowski and Ramsby 2003).

6.1.3 The origin and regulation of circulating MMP-8

In addition to *CFH* polymorphism, genetic variation in the locus containing genes for S100 calcium binding proteins A8, A9, and A12 was associated with serum MMP-8 levels. The minor allele of the SNP rs1560833, which is located downstream of *S100A9*, was associated with a lower concentration of MMP-8 in both serum and plasma. According to the eQTL analysis, the minor allele of rs1560833 has an inverse association with the expression of *S100A8*, *S100A9*, and *S100A12*. S100A8, S100A9, and S100A12 are proinflammatory mediators expressed mainly by the neutrophils. They activate Toll-like receptors, induce cytokine secretion, and enhance particularly the innate immune response (Sunahori et al. 2006, Foell et al. 2007, Vogl et al. 2007, Riva et al. 2012). In the presence of calcium, S100A8 and S100A9 form a S100A8/S100A9 complex known as calprotectin. S100A8, S100A9, S100A8/A9, and S100A12 can induce neutrophil recruitment, chemotaxis, and adhesion (Newton and Hogg 1998, Ryckman et al. 2003, Vandal et al. 2003, Pruenster et al. 2015). In addition, S100A9 has been reported to stimulate the degranulation of neutrophils (Simard et al. 2010). Therefore, genetic polymorphisms that affect the expression of the S100A proteins may influence circulating MMP-8 concentrations by regulating neutrophil activity and degranulation.

A previous study that used a relatively small study population suggested that a SNP in the promoter region of *MMP8*, rs11225395, was associated with serum MMP-8 concentration (Pradhan-Palikhe et al. 2012). In our study, the SNPs in the *MMP8* gene had no association with MMP-8 levels in serum. As the major polymorphisms that affected serum MMP-8

were located in the loci for *CFH* and *S100A8/A9/A12*, it seems that the main mechanism that controls the concentration of MMP-8 is the regulation of neutrophil degranulation, rather than the gene expression of *MMP8 per se*.

Even though the polymorphism of *CFH* was not associated with plasma MMP-8, the carriers of the CFH Ile62 variant are likely to have less "activation potential" for the alternative pathway of complement. Therefore, the Ile62 variant is likely to protect from diseases that are characterized by excess activation of the complement system. In previous studies, the CFH Ile62 variant has been associated with a decreased risk for age-related macular degeneration, which is a disease characterized by dysregulation of local inflammatory response (Hageman et al. 2005). As complement activation seems to be an important inducer of neutrophil degranulation and MMP-8 release, the Ile62 variant may also reduce susceptibility to diseases characterized by excess MMP-8 activity, such as periodontitis or rheumatoid arthritis.

6.1.4 MMP-8-associated genetic polymorphisms and the risk of cardiovascular diseases

The analysis of FINRISK cohort studies and follow-up data revealed that the minor allele of rs1560833 was associated with a lower risk of prevalent and incident CVD events in men, but not in women. Since the association was relatively weak and seen only in men, a replication of this finding in other populations is needed to verify the result. *CFH* polymorphisms were not associated with CVDs in our population. As S100A8, S100A9, and S100A12 are proinflammatory mediators, their role in the pathogenesis of CVDs and their value as biomarkers of CVDs has been investigated in several studies. S100A8/A9 is expressed by neutrophils and macrophages in atherosclerotic lesions, and it is regarded as a marker of plaque instability (Miyamoto et al. 2008, Ionita et al. 2009). Elevated serum concentration of S100A8/A9 is associated with ACS and AMI (Altwegg et al. 2007, Schaub et al. 2012, Vora et al. 2012), as well as with the risk of a first cardiovascular event in the general population, and also with a recurrent cardiovascular event in ACS patients independently of traditional risk factors (Healy et al. 2006, Morrow et al. 2008, Cotoi et al. 2014). The plasma concentration of S100A8/A9 is also positively associated with neutrophil counts (Cotoi et al. 2014). Like S100A8/A9, MMP-8 is also associated with plaque vulnerability and the prevalence and incidence of CVDs. However, the actual relationship

between these two markers and CVD remains unclear. The genetic variation of S100A8/A9/A12 might be related to CVD via MMP-8, or, alternatively, S100A proteins and MMP-8 may each have an independent role in the inflammatory processes contributing to CVDs.

6.2. Processing of apoA-I by MMP-8

6.2.1 MMP-8 cleaves apoA-I and reduces the cholesterol efflux capacity of HDL

The most commonly known function of MMP-8 is to process and remodel the ECM, its main substrate being collagen type I. In addition to ECM components, MMP-8 has numerous other bioactive substrates, e.g. cytokines, chemokines, receptors, and signaling molecules. The cleavage of these substrates may have a substantial influence on a wide range of physiological processes, such as the inflammation response, signaling pathways, and metabolic pathways. We discovered that MMP-8 degrades human apoA-I and apoA-II molecules. The cleavage of apoA-I occurred at the C-terminus of the protein, which is, according to several previous studies, especially susceptible to proteolysis (Kunitake et al. 1990, Dalton and Swaney 1993, Ji and Jonas 1995, Lindstedt et al. 1999). The C-terminal domain of apoA-I is essential for the ability of the protein to promote cholesterol and phospholipid efflux from cells (Sviridov et al. 1996, Favari et al. 2002, Chroni et al. 2003). Our results show that MMP-8 significantly decreased the capacity of apoA-I and HDL to facilitate cholesterol efflux from cholesterol-loaded human THP-1 macrophages, which were used as a model of foam cells in atherosclerotic lesions.

The conformation and properties of apoA-I vary significantly between different HDL subpopulations (Jonas et al. 1990, Huang et al. 2011). ApoA-I in lipid-free state or within pre-β HDL is proteolyzed more easily than apoA-I in HDL with α-mobility (Kunitake et al. 1990, Lindstedt et al. 1999, Lindstedt et al. 2003). In our experiments, we did not see proteolysis of apoA-I within HDL_2 or HDL_3 fractions when they were analyzed by SDS PAGE. However, MMP-8 significantly decreased the cholesterol efflux capacity of both HDL_2 and HDL_3. We did not analyze the amino acid sequence of HDL-associated apoA-I after treatment with MMP-8. Even very small changes in the structure and subclass distribution of HDL particles may cause a significant decrease in their ability to facilitate cholesterol efflux from foam cells (Lindstedt et al. 2003). Such changes may not be

detectable in SDS PAGE. Lipid-poor apoA-I, pre-β HDL, and very small HDL particles are the first and most efficient acceptors of cholesterol from cells (Du et al. 2015), and their degradation could have significant effects on HDL metabolism and reverse cholesterol transport.

6.2.2 The role of MMP-8 in atherosclerosis

The most studied role of MMP-8 in atherosclerosis arises from the degradation of the fibrous cap in atherosclerotic lesions, and consequent changes in plaque stability. However, our results indicate that MMP-8 may also contribute to atherosclerosis by processing non-collagenous substrates. Proteolytic modification of apoA-I and apoA-II, and potentially other HDL proteins, by MMP-8 and the resulting reduction in the cholesterol efflux capacity of HDL may impair the first steps of reverse cholesterol transport, which could lead to the increased accumulation of foam cells in the vessel wall. These modifications may result in accelerated atherogenesis.

The major source of MMP-8 in tissues is degranulating neutrophils. The role of neutrophils in the pathogenesis of atherosclerosis has not been as widely studied as that of e.g. monocytes/macrophages. Analysis of lesions in coronary arteries has shown that ruptured plaques contain significant numbers of neutrophils, suggesting that neutrophil infiltration is associated with acute coronary syndrome (Dinerman et al. 1990, Naruko et al. 2002). High neutrophil counts are also found in rupture-prone carotid artery plaques (Ionita et al. 2010). An *in vitro* study found that LDL accumulation stimulates the adherence, transmigration, and local infiltration of PMN cells into the intima (Dorweiler et al. 2008). The infiltrating PMN released MMP-8 in response to LDL (Dorweiler et al. 2008). In addition to neutrophils, other cell types are also capable of producing MMP-8. At least macrophages, endothelial cells, and smooth muscle cells express MMP-8 within atherosclerotic lesions, whereas MMP-8 was not recovered from normal arteries (Herman et al. 2001b, Molloy et al. 2004). The concentration of MMP-8 in relation to apoA-I might be high in vulnerable plaques and especially near the surface of the cells expressing MMP-8. The levels of MMP-8 in the circulation have a wide range of variation and also show significant variation between individuals. Patients with cardiovascular disorders tend to have higher

concentrations of MMP-8 and lower concentrations of HDL. These higher MMP-8:apoA-I ratios may accelerate the proteolysis of apoA-I in the circulation.

We found that MMP8$^{-/-}$ mice had lower serum TG concentrations and larger HDL particle size compared to WT mice. However, the serum cholesterol efflux capacities or apoA-I levels did not differ between the mouse groups. It should be noted that healthy young mice fed on a normal diet do not develop significant atherosclerosis or high blood concentrations of MMP-8. The cleavage of apoA-I by MMP-8 within the atherosclerotic plaques or in individuals with significantly high circulating MMP-8 levels, such as patients with cardiovascular diseases, cannot be detected in this mouse model. The observed shift of HDL size towards larger particles in MMP8$^{-/-}$ mice and the difference in serum TG levels between the mouse groups indicate that MMP-8 might participate in HDL modification and also in overall lipoprotein metabolism. The MMP-8 knockout mouse model offers numerous opportunities for future research on the mechanisms by which MMPs contribute to atherosclerosis. However, direct conclusions concerning humans cannot be made on the basis of the mouse study because of the fundamental differences in lipoprotein metabolism between mice and humans; mice do not have CETP, and therefore the major carrier of cholesterol in mice is HDL.

6.3 Potential effects of doxycycline therapy on lipoprotein metabolism

6.3.1 Doxycycline as MMP inhibitor in atherosclerosis

Tetracyclines have, in addition to their well-known antimicrobial activity, also inhibitory effects against MMPs. Therefore, the members of the tetracycline family could stabilize unstable, inflamed atherosclerotic plaques and prevent acute coronary events by inhibiting the action of MMPs (Bench et al. 2011). A placebo-controlled randomized clinical trial with doxycycline before carotid endarterectomy showed that doxycycline significantly reduced the concentration of MMP-1 in carotid plaques of patients (Axisa et al. 2002). Doxycycline given to rats reduced the activities of MMPs 2 and 9 in the arterial wall after arterial injury, and it also inhibited the migration of smooth muscle cells from media to intima (Bendeck et al. 2002). The concentration of MMP-8 was not measured in those two studies, but it is plausible that doxycycline treatment also affects MMP-8 activity within the atherosclerotic

plaques. Our study suggests that doxycycline could increase the cholesterol efflux potential of apoA-I by inhibiting the proteolytic modification by MMP-8.

The mean concentration of doxycycline within carotid plaques was 6.0 µg/g wet weight in patients treated with doxycycline (Axisa et al. 2002).,Doxycycline levels in the circulation of patients ranged from 1.3 to 14.4 µg/ml after three months of doxycycline treatment (Baxter et al. 2002). The concentration of 10 µg/ml corresponds to approx. 22.5 µM doxycycline. In order to achieve 50% inhibition of MMP-8 activity, a concentration of 15-30 µM of doxycycline is required (Suomalainen et al. 1992). Therefore, the clinically attainable concentrations of doxycycline and the concentrations used in our experiments are relevant for the inhibition of MMP-8.

6.3.2 Systemic effects of SDD treatment

Doxycycline at low doses has no antimicrobial property, but maintains its inhibitory effect against MMPs. Treatment with SDD therefore does not result in the development of antibiotic resistance, and therefore the treatment can be continued for relatively long time periods. Earlier studies report that SDD has beneficial effects on inflammatory markers in blood.A six-month SDD therapy in patients with symptomatic CAD lowered the plasma levels of hsCRP by 46% (Brown et al. 2004). In addition, IL-6 concentration and MMP-9 activity decreased in the treatment group (Brown et al. 2004). Two years of treatment with SDD decreased serum hsCRP and MMP-9 levels also in a group of postmenopausal women that had periodontitis (Payne et al. 2011). In our placebo-controlled clinical trial, mean change in the cholesterol efflux capacity of serum was significantly higher among the SDD treated group compared to placebo group after adjustment for baseline cholesterol efflux levels and smoking.The cholesterol efflux levels in SDD subjects were significantly increased at the 1-year and 2-year time points relative to baseline. However, there was also an increase in mean cholesterol efflux levels in the placebo group at the 2-year time point, although this was not statistically significant. The increased cholesterol efflux observed among placebo patients may be due to statin medication: three of the placebo patients either started statin medication or increased the dose during the trial compared to none of the SDD patients, which may affect the results in this relatively small sample. Studies with larger populations and with both genders are needed to confirm our results.

Inflammation causes significant changes in the composition and metabolism of lipoproteins. The antiatherogenic potency of HDL is diminished, and the levels of HDL cholesterol and apoA-I are decreased during both acute and chronic inflammations (Khovidhunkit et al. 2000, Khovidhunkit et al. 2004, Pussinen et al. 2004c). We did not observe changes in the concentrations of apoA-I or HDL cholesterol during SDD treatment. However, we did not analyze the detailed composition or subclass distribution of HDL. SDD therapy attenuates systemic inflammation, and, according to our results, improves the ability of serum to act as a cholesterol acceptor. In addition, doxycycline protects apoA-I from MMP-induced degradation. Therefore, doxycycline at both regular and low doses might reduce the risk of cardiovascular disease.

6.4 Salivary diagnostics of periodontitis

6.4.1 Biomarkers of periodontitis

Biomarkers of periodontitis include host-derived markers, such as cytokines, MMPs, antibodies, or byproducts of tissue breakdown, and pathogen-derived markers, such as bacterial DNA or LPS. We measured the concentrations of three biomarkers, each of which represented a distinct component of the pathogenesis of periodontitis: MMP-8 as a marker of the host response, IL-1β as a marker of systemic inflammation, and *P. gingivalis* as a representative of the pathogen burden and a keystone pathogen.

Increased levels of MMP-8 have been found in the saliva, GCF, and mouthrinse of periodontitis patients in a large number of studies (Gangbar et al. 1990, Miller et al. 2006, Mäntylä et al. 2006, Christodoulides et al. 2007, Gursoy et al. 2010, Leppilahti et al. 2011, Ebersole et al. 2013, Rathnayake et al. 2013). It is widely accepted that MMP-8 has a major role in the tissue destruction associated with periodontitis. In addition, MMP-8 may modulate the host immune response by cleaving cytokines and chemokines. In our study, the concentration of MMP-8 in salivawas associated with ABL and the number of deepened periodontal pockets. In addition, MMP-8 had the strongest association with BOP among the markers we examined. BOP is considered as a sign of active inflammation. Therefore, MMP-8 seems to be associated with current, active periodontitis.

IL-1β is a key mediator of APR and chronic inflammation. Previous studies indicate that it is a sensitive and specific biomarker of periodontal disease (Sánchez et al. 2013). In our

study, the salivary concentration of IL-1β was strongly associated with the number of deepened periodontal pockets (PPD 4-5 mm or ≥ 6 mm) and a high PIBI index, which reflects the periodontal inflammatory burden. The association with ABL was not as strong. Concordantly, Zhong et al. showed in a large American study that the concentration of IL-1β in GCF was associated with deep periodontal pockets and BOP, but not with attachment loss (Zhong et al. 2007). Loss of bone and attachment might reflect the total history of periodontitis instead of current, active disease, which could explain the weaker association with IL-1β, a marker of current inflammatory activity.

Higher numbers of *P. gingivalis* have been detected in the saliva of periodontitis patients compared to controls previously (Hyvärinen et al. 2009, Saygun et al. 2011). Our study indicates that the salivary number of *P. gingivalis* was associated with the number of deepened periodontal pockets and the extent of ABL, but not with BOP. However, as a keystone pathogen, *P. gingivalis* can significantly contribute to the pathogenesis of periodontitis even at low abundance, and therefore its concentration might give slightly inconclusive results.

We used stimulated saliva samples for biomarker analyses in our study. Saliva can be obtained without stimulation or after stimulation of secretion by chewing. According to Golatowski et al., the saliva collection techniques of drooling, use of cotton swabs (Salivette) or stimulation by chewing of paraffin gum resulted in similar protein concentrations and numbers of identified proteins in saliva, whereas minor differences were observed only in the volume of saliva samples obtained (Golatowski et al. 2013). In addition, the microbial profiles of unstimulated and stimulated saliva samples are similar (Belstrøm et al. 2016). Therefore, it is unlikely that the method of obtaining the saliva sample has significant effects on the diagnostic value of our biomarkers.

The European Federation of Periodontology has stated that attachment loss should be the primary measure when defining periodontitis in epidemiological studies (Tonetti et al. 2005). In addition, markers of current disease activity, such as BOP or PPD, should be recorded. Similarly, the American Academy of Periodontology recommends that periodontitis should be defined based on attachment loss and PPD (Eke et al. 2012).We defined periodontitis in the Parogene study basing it on ABL and PPD. As the location of the cementoenamel junction or the extent of gingival recession was not recorded in our clinical examination, we were not able to determine clinical attach loss for each tooth. It

should be noted that a strict definition of "periodontitis case" is not important in clinical practice because the aim is to identify the individuals who need treatment or are at risk of developing the disease. The studies on periodontal biomarkers are often case-control investigations, with patients having established periodontitis as cases and totally healthy individuals as controls. The "borderline" cases or cases with mild periodontitis are often excluded, even though they are likely to complicate the diagnostics in reality. The population of our study was not selected on the basis of periodontal status. Our study participants represented all stages of periodontal conditions from periodontally healthy subjects to severe periodontitis patients.

6.4.2 The cumulative approach

Periodontitis is a complex disease with a multifactorial aetiology. The fluctuation in the progression and activity of the disease may influence the diagnostic power of the salivary biomarker selected (Gursoy et al. 2011). For example, a certain biomarker may be present at a low concentration during the remission period of the disease. It is unlikely that a single biomarker could be used for the optimal detection and risk assessment of periodontitis. Two previous studies have indicated that a combination of plaque pathogens and salivary biomarkers is more strongly associated with concurrent periodontitis and the progression of the disease than individual markers (Ramseier et al. 2009, Kinney et al. 2011). In our study, we tested the validity of a novel diagnostic tool, the cumulative risk score for detection of periodontitis. The CRS index, a combination of the three biomarkers that represent the pathogen / tissue destruction / systemic inflammation cascade of periodontitis, had a stronger association with periodontitis than any of the markers individually. Therefore, it offers a tool for detection of periodontitis in a non-invasive way.

6.4.3 Systemic diseases and salivary diagnostics

Saliva has gained wide interest as a diagnostic fluid of periodontitis because saliva samples can be easily, non-invasively, and repeatedly obtained. Saliva has potential especially for large-scale studies, health care promotion, home testing, and screening for high-risk individuals in the general population. Various systemic factors, such as smoking, rheumatoid arthritis, or diabetes, may affect the concentrations of biomarkers in saliva

(Liede et al. 1999, Furuholm et al. 2006, Costa et al. 2010, Mirrielees et al. 2010, Rathnayake et al. 2013, Sorsa et al. 2016), which complicates the use of saliva in periodontal diagnostics. A good diagnostic marker should be able to differentiate periodontitis patients from healthy individuals and to evaluate disease activity and progression regardless of systemic factors, as periodontitis patients are often smokers and elderly people with additional diseases. The salivary concentration of IL-1β in our study was higher in individuals with stable CAD or ACS compared to those with no CAD. However, the concentrations of MMP-8 and *P. gingivalis* or the CRS index scores did not differ between the CAD subgroups. A Swedish study of 200 patients with myocardial infarction and 200 controls consistently found that saliva MMP-8 was significantly associated with periodontal status, but could not differentiate individuals with or without myocardial infarction (Rathnayake et al. 2015). It seems therefore that salivary MMP-8 mostly reflects the health of the oral cavity, whereas the concentrations of MMP-8 in the circulation are associated with the CAD status of the individuals.

7. CONCLUSIONS

This thesis examined the following topics: (1) genetic variants and regulatory mechanisms that affect serum MMP-8 concentrations, (2) the effect of MMP-8 on the structure and function of apoA-I and HDL, (3) the potential of doxycycline in inhibiting the MMP-8-mediated cleavage of apoA-I, (4) the effect of SDD treatment on the cholesterol efflux capacity of serum, and (5) salivary biomarkers in diagnostics of periodontitis in patients with cardiovascular disorders.

The results of our genome-wide association study and functional experiments indicate that the activation of the complement system, especially its alternative pathway with amplification potential, strongly contributes to serum MMP-8 concentrations. The association between genetic polymorphism of complement factor H and serum MMP-8 was discovered in two independent populations and in the meta-analysis that combined them. In addition to the genetic variation of complement factor H, polymorphism in the genetic locus of *S100A8/A9/A12* affects circulating MMP-8 concentrations, possibly via neutrophil activation and degranulation. These results suggest that the main regulatory mechanism of circulating MMP-8 levels is neutrophil degranulation, rather than the regulation of *MMP8* gene expression, since *MMP8* promoter polymorphisms that affect its mRNA expression were not associated with circulating MMP-8 levels. In large population-based cohorts, genetic polymorphism in the *S100A8/A9/A12* region was associated with a decreased risk for CVD in men. The complement system is an integral part of innate immunity and *S100A8*, *S100A9*, and *S100A12* are inflammatory mediators. Our results therefore emphasize the importance of inflammation and the innate immune system in cardiovascular disorders.

Incubation of apoA-I with MMP-8 resulted in a two-step cleavage of the C-terminus of apoA-I. In addition, the ability of apoA-I and HDL to promote cholesterol efflux from cholesterol-loaded macrophages was significantly diminished after pre-treatment with MMP-8. When we used an MMP-8 knockout mouse model, we found that MMP-8 knockouts displayed lower concentrations of TGs in serum and larger HDL particles compared to wild-type mice. Our findings suggest that proteolytic modification of apoA-I by MMP-8 and the reduction in the cholesterol efflux capacity of HDL may impair the first steps of reverse cholesterol transport. As a result, increased accumulation of cholesterol in the vessel wall may lead to accelerated atherosclerosis. Our results introduce a new substrate

for MMP-8 and suggest that MMP-8 may affect the metabolism of lipids and lipoproteins. The study indicates that the role of MMPs in atherosclerosis may not be limited to the processing of the ECM.

Degradation of apoA-I by MMP-8 was inhibited by doxycycline at clinically attainable doses. Doxycycline was also able to restore the cholesterol efflux capacity of apoA-I after treatment with MMP-8. According to our findings, MMPs are a potential target for medication in cardiovascular disorders.

In the placebo-controlled clinical trial with SDD medication, the cholesterol efflux capacity of serum increased 8.1% over the first study year in the SDD treatment group. Such an increase may have relevant beneficial effects on the development and progression of atherosclerosis as cholesterol efflux from cells is the first and rate-limiting step of reverse cholesterol transport.. SDD medication increased the cholesterol efflux capacity of serum particularly in the subgroup of women who were more than five years postmenopausal. Periodontitis patients display lower levels of apoA-I and HDL cholesterol, and periodontitis causes proatherogenic changes in the composition of HDL particles. SDD therapy may reduce the risk of cardiovascular disease especially in vulnerable groups of individuals, such as periodontitis patients with "dysfunctional" HDL, or postmenopausal women, who have reduced levels of protective estrogen.

Our study of patients who had angiographically verified cardiac diagnosis showed that salivary concentrations of MMP-8, IL-1β, and *P. gingivalis* are associated with periodontitis and various clinical and radiographic periodontal parameters. The cumulative risk score, combining the three salivary biomarkers that reflect the distinct stages of the disease, had a stronger association with moderate to severe periodontitis than any of its constituent markers alone. Salivary biomarkers detected periodontal conditions regardless of the systemic disease of the patients. Salivary diagnostics of periodontitis therefore has potential especially in large population studies in which detailed clinical examinations are not feasible.

To summarize, we found new mechanisms that contribute to the concentrations of MMP-8 in serum and plasma, and a suggestive association between a novel genetic polymorphism and CVD. We identified a new substrate for MMP-8, and thereby revealed a new mechanism by which MMP-8 may be linked to cardiovascular disorders. We also confirmed

that MMP-8 is a potential target of medication in cardiovascular disorders. In addition, we found that salivary MMP-8 and the cumulative risk score reflect the periodontal conditions, but not CAD status of the patients, whereas serum MMP-8 is associated with the CAD status. Our results suggest that MMP-8 functions as a link between inflammatory disorders, such as periodontitis, and cardiovascular disorders.

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