Lipopolysaccharide: a link between periodontitis and cardiometabolic disorders

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Lecture Hall 2, Biomedicum 1, Haartmaninkatu 8, Helsinki, on December 12th 2014, at 12 noon.

Helsinki 2014

ISBN 978-951-51-0458-8 (paperback)
ISBN 978-951-51-0459-5 (PDF)
ISSN 2342-3161 (Print)
ISSN 2342-317X (Online)
http://ethesis.helsinki.fi
Unigrafia
Helsinki 2014

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LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original publications, referred to in the text by their Roman numerals I–IV.

I. Kallio KA*, Marchesani M*, Vlachopolou E, Mäntylä P, Paju S, Buhlin K, Suominen AL, Contreras J, Knuuttila M, Hernandez M, Huumonen S, Nieminen MS, Perola M, Sinisalo J, Lokki ML, Pussinen PJ. Genetic variation on the BAT1-NFKBIL1-LTA region of major histocompatibility complex class III associates with periodontitis. *Infection and Immunity* 2014 May; 82(5):1939-48.

II. Kallio KA, Buhlin K, Jauhiainen M, Keva R, Tuomainen AM, Klinge B, Gustafsson A, Pussinen PJ. Lipopolysaccharide associates with pro-atherogenic lipoproteins in periodontitis patients. *Innate Immunity* 2008 Aug; 14(4):247-53.

III. Kallio KA, Hyvärinen K, Kovanen PT, Jauhiainen M, Pussinen PJ. Very low density lipoproteins derived from periodontitis patients facilitate macrophage activation via lipopolysaccharide function. *Metabolism* 2013 May; 62(5):661-8.

IV. Kallio KA, Hätönen KA, Lehto M, Salomaa V, Männistö S, Pussinen PJ. Endotoxemia, nutrition, and cardiometabolic disorders. *Acta Diabetologica* 2014 Oct 19 (Epub ahead of print).

* The authors contributed equally to the study.
In addition, this thesis contains some unpublished data.

ABBREVIATIONS

AAP	American Academy of Periodontology
ABC	ATP-binding cassette transporter
ABL	Alveolar bone loss
ACAT-1	Acetyl-Co A acetyltransferase 1
AMI	Acute myocardial infarction
apo	Apolipoprotein
ACS	Acute coronary syndrome
BAT1	HLA-B-associated transcript 1
BMI	Body mass index
BOP	Bleeding on probing
CAD	Coronary artery disease
CAL	Clinical attachment level
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
ECM	Extracellular matrix
EFP	European Federation of Periodontology
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin unit
FA	Fatty acid
FFA	Free fatty acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCF	Gingival crevicular fluid
GGT	Gamma-glutamyltransferase
GWA	Genome-wide association
HDL	High-density lipoprotein
HWE	Hardy–Weinberg equilibrium
IDF	International Diabetes Federation
IDL	Intermediate-density lipoprotein
IL	Interleukin
IQR	Interquartile range
LAL	*Limulus* amebocyte lysate
LBP	Lipopolysaccharide binding protein
LCAT	Lecithin-cholesterol acyltransferase

LDL	Low-density lipoprotein
Lp(a)	Lipoprotein(a)
LPDP	Lipoprotein deficient plasma
LPS	Lipopolysaccharide
LTA	Lymphotoxin-α
MetS	Metabolic syndrome
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MUFA	Monounsaturated fatty acid
NAFLD	Non-alcoholic fatty liver disease
nCEH	Neutral cholesterol ester hydrolase
NFκB	Nuclear factor-κB
NFKBIL1	Nuclear factor of κ light chain gene enhancer in B cells inhibitor-like 1
OR	Odds ratio
PAL	Proximal attachment loss
PBS	Phosphate-buffered saline
PPD	Probing pocket depth
PL	Phospholipid
PLTP	Phospholipid transfer protein
PMA	Phorbol 12-myristate 13-acetate
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative real-time polymerase chain reaction
SAA	Serum amyloid A
SFA	Saturated fatty acid
SNP	Single nucleotide polymorphism
SR	Scavenger receptors
SR-B1	Scavenger receptor class B, member 1
TG	Triglyceride
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T2DM	Type 2 diabetes mellitus
VLDL	Very low-density lipoprotein
WHO	World Health Organization

ABSTRACT

Periodontitis is characterized by an inflammatory response to bacterial infection in the supporting tissues of the teeth. The disease manifests with gingival swelling and bleeding, increased periodontal pocket depth, and alveolar bone loss. Intact bacteria or bacterial products, including lipopolysaccharide (LPS), may enter the bloodstream through inflamed periodontal tissue or via saliva. Bacterial dissemination, further potentiated by gastrointestinal microbiota, may result in endotoxemia and low-grade inflammation.

The general aim of this thesis research was to investigate whether LPS links periodontitis with cardiometabolic disorders. The following topics were studied: genetic factors associated with the susceptibility to periodontitis, the systemic effects of endotoxemia induced by periodontitis and cardiometabolic disorders, as well as the influence of periodontal treatment on plasma LPS activity and lipoprotein composition.

A study of genetic polymorphisms of the human major histocompatibility complex region demonstrated that a haplotype comprising six SNPs of the *BAT1*, *NFKBIL1*, and *LTA* genes was associated with the risk of having periodontitis. The risk haplotype showed an association with bleeding on probing, probing pocket depth ≥6 mm, and severe periodontitis, and the result was replicated in two different study populations with concordance. In addition, the serum lymphotoxin-α (LTA) concentration was associated with *LTA* SNPs of the risk haplotype in homozygous patients, and LTA was expressed in the inflamed periodontal tissue.

The systemic effects of the periodontitis-derived endotoxemia were investigated before and after periodontal treatment. In the serum of periodontitis patients, LPS was associated with the proatherogenic very low-density lipoprotein – intermediate-density lipoprotein (VLDL-IDL) fraction. Although local healing of the periodontium was successful, the systemic inflammation status of the patients failed to improve after periodontal treatment, reflecting the complexity and persistence of the disease. There were no significant changes in plasma LPS activity or its distribution among lipoprotein classes after periodontal treatment. However, the VLDL of patients with severe periodontitis induced higher expression of proinflammatory cytokines in macrophages when compared with VLDL derived from patients with moderate periodontitis. In addition, VLDL isolated from patients with severe periodontitis with suppuration contained more LPS and induced higher cholesterol uptake in macrophages.

The effect of nutrient intake on the association of serum LPS activity with cardiometabolic disorders was examined in a population-based cohort. Endotoxemia was strongly associated with prevalent obesity, metabolic syndrome (MetS), diabetes, and coronary

heart disease (CHD). In addition, high serum LPS activity was associated with an increased risk of future CHD events. Even though energy intake was correlated with LPS activity in lean, healthy subjects, the general associations were independent of energy or macronutrient intake.

The results indicate that genetic variation in the MHC class III region may be important in periodontitis susceptibility. Endotoxemia and low-grade inflammation originating from periodontitis may induce the proatherogenic properties of VLDL particles via macrophage activation and foam cell formation, thereby promoting atherogenesis. The association of obesity, MetS, diabetes, and CHD with endotoxemia supports the significance of bacterial infections and the immune response in the etiology of cardiometabolic disorders. In conclusion, the findings highlight the close relationship between genetics, the immune response, and lipid metabolism, promoting the role of LPS as a link between periodontitis and cardiometabolic disorders.

Keywords: periodontal disease, genetics, lipopolysaccharide, lipoproteins, treatment, cardiometabolic disorders, nutrition

1. REVIEW OF THE LITERATURE

1.1. Periodontal disease

Periodontitis is an inflammatory disease of the supporting tissues of the teeth initiated by microorganisms, resulting in progressive destruction of the periodontal ligament and bone support. The host response to bacterial insult leads to inflammatory gingival swelling and bleeding from the gingival pocket on gentle probing, increased pocket depth, recession, or both, and alveolar bone loss. Finally, untreated periodontitis may lead to the loss of teeth. It is among the most common causes of tooth loss worldwide.

1.1.1. Structure of the periodontium

Healthy periodontal tissue is composed of four principal components: gingiva, periodontal ligament, root cementum, and alveolar bone (**Figure 1**). The gingiva covers the alveolar bone and tooth root to a level just coronal to the cementoenamel junction. The gingival epithelium is morphologically and functionally divided into the oral epithelium, junctional epithelium, and sulcular epithelium. The shallow, V-shaped region between the tooth and the sulcular epithelial surface is called the sulcus. In periodontitis, the volume of sulcular fluid or the gingival crevicular fluid (GCF) increases. GCF is an inflammatory exudate composed of serum and locally produced molecules such as inflammatory mediators, antibodies, and tissue breakdown products. In addition to saliva, it offers potential use as a sample material for diagnostics or prognostics when analyzing the health status of the periodontium (Embery et al. 2000). The probing depth of a healthy gingival sulcus is 2-3 mm (Newman et al. 2012). The fibrous connective tissue structure, periodontal ligament, joins the root to the alveolar bone. One side of the periodontal ligament is attached to the root cementum and the other side to the alveolar bone. It serves as a shock absorber by mechanisms that provide resistance against physical forces and participates in the repair and resorption of cementum and bone, and supplies nutrients to the periodontium.

The periodontal pocket, denoting a deepened sulcus provoked by bacterial plaque, is one of the most important clinical and pathological changes associated with periodontal disease (**Figure 1**) (Newman et al. 2012). The clinical attachment level (CAL) represents the distance from the cementoenamel junction of the tooth to the bottom of the pocket, and it often correlates with periodontal pocket depth. The destruction of the supporting periodontal tissue can involve one or more tooth surfaces.

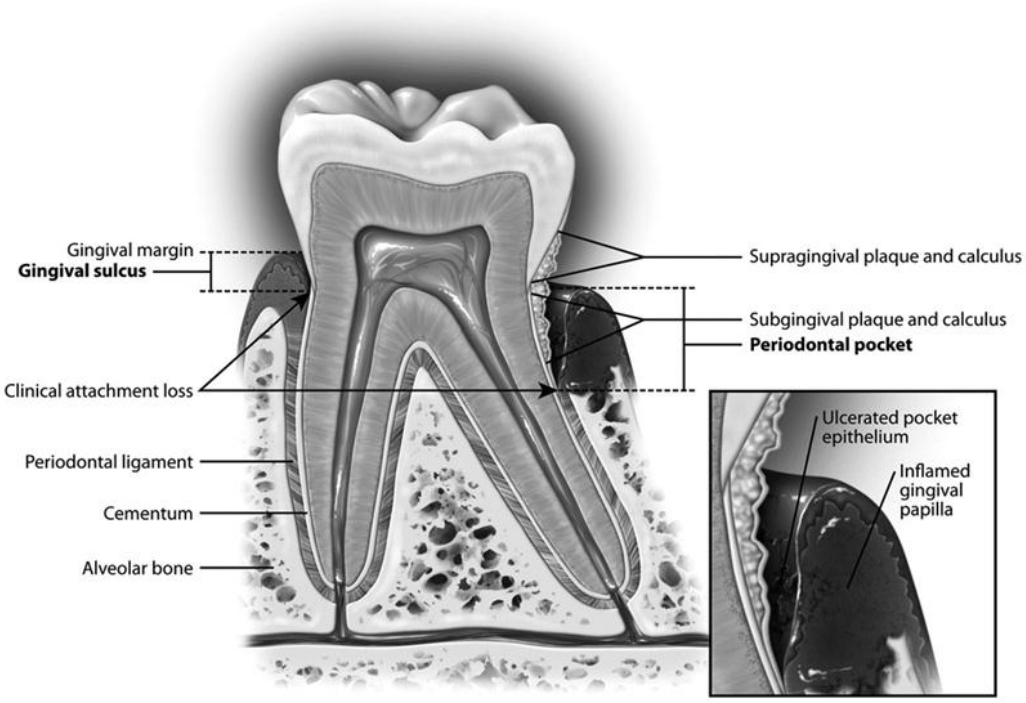

Figure 1. Periodontal anatomy and the effects of periodontitis. The left side of tooth represents the healthy periodontal tissue and the right side the presence of periodontal disease. (Lockhart et al. 2012) Reprinted with permission from Wolters Kluwer Health.

1.1.2. Classification of periodontal disease

Gingivitis is a reversible form of periodontal disease with increased GCF flow and swelling and redness of the gingiva, which without the treatment may lead to periodontitis. The classification system for periodontal diseases established in 1999 listed the following major categories of destructive periodontal diseases: 1) chronic periodontitis, 2) aggressive periodontitis, 3) periodontitis as a manifestation of systemic disease, 4) necrotizing ulcerative gingivitis / periodontitis, 5) abscesses of the periodontium, and 6) combined periodontic-endodontic lesions (Armitage 2004), from which chronic periodontitis and aggressive periodontitis are described here in more detail.

The most common form of periodontitis among the adult population is chronic periodontitis. It occurs as a slowly progressing disease. The clinical findings generally include supra- and subgingival plaque accumulation associated with the formation of dental calculus, gingival inflammation, periodontal pocket formation, loss of tooth attachment, and occasional suppuration. Chronic periodontitis is a common disease worldwide and the prevalence increases with age in both genders. In the United States, over 47% of the adult population suffers from periodontitis (Eke et al. 2012), while in

Finland, 64% of adults have deepened periodontal pockets and 21% are diagnosed with more severe forms of the disease (Knuuttila and Suominen-Taipale 2008).

Aggressive periodontitis differs from the chronic form primarily by the rapid destruction of the periodontal ligament and alveolar bone in otherwise healthy individuals. There is an absence of notable accumulations of plaque and calculus, while otherwise the clinical findings may be similar to those observed in chronic periodontitis. A family history of aggressive periodontitis has been acknowledged as suggestive of a genetic trait (Vieira and Albandar 2014) (see also 1.1.5.). Clinically, aggressive periodontitis may occur either as localized disease or as generalized disease. Localized aggressive periodontitis generally has a circumpubertal onset, while patients with generalized aggressive periodontitis are typically - but not necessarily - under the age of 30 years (Lang et al. 1999). The prevalence of aggressive periodontitis varies greatly among different ethnic groups from ≤0.5% in a Caucasian population to 1–5% in African populations (Susin et al. 2014). In Finland, the prevalence of juvenile periodontitis (an old term replaced by aggressive periodontitis since 1999) has been reported to situate between 0.06 and 0.26% (Saxen 1980). Currently, many parts of the world still lack information on the epidemiology of the disease.

1.1.3. Pathogenesis of periodontitis

The onset of periodontitis is characterized by inflammation of the gingiva in response to bacterial challenge. Information based on the application of massively parallel pyrosequencing linked to 16S rDNA analysis has increased the estimated number of bacterial phylotypes in the oral cavity to 2×10^4 (Keijser et al. 2008), and the developing techniques are continuously identifying novel bacteria associated with periodontal pocket depth. As periodontitis proceeds, the bacterial composition of the overgrowing subgingival biofilm transforms from the dominance of Gram-positive bacteria to a majority of Gram-negative bacteria (Marsh 1994). Socransky et al. (1998) contributed to further understanding of the different bacteria associated with periodontal disease by revealing five major microbial color-coded complexes identified with DNA-DNA hybridization. These sets of bacteria were repeatedly found together in periodontitis. *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* were determined to form the 'red-complex' periopathogens, since they had a strong association with periodontitis-related variables, for example periodontal pocket depth (Socransky et al. 1998). In addition, *Aggregatibacter actinomycetemcomitans* is among the key bacteria implicated in the pathology of periodontal disease (Henderson et al. 2010; Könönen and Müller 2014).

Although periodontopathic bacteria are needed for the initiation of periodontitis, the volume of plaque and the bacterial species do not alone correlate with the severity of the

disease (Offenbacher et al. 2008). Several systemic and local risk factors are involved in modifying the susceptibility or resistance to the periodontitis. The common risk factors in addition to age, gender, ethnicity, and genetic factors include lifestyle and human behavior, such as smoking and alcohol, and medical conditions, such as dyslipidemia, diabetes, obesity, osteoporosis, and stress (Bouchard et al. 2006; Könönen et al. 2010; Genco and Borgnakke 2013), although other risk factors, for example a low educational level (Boillot et al. 2011), have also been identified. Therefore, it is important for clinicians to search for risk factors beyond the oral cavity in order to understand the complex nature of periodontal disease.

1.1.4. Host response

Periodontitis is described as polymicrobial disruption of host homeostasis (Darveau 2010). Pathogenic biofilms cause a challenge to the host response; therefore, the immune system has a substantive role in the maintenance of periodontal health. The different microbial- and host-derived markers of periodontitis can be measured locally from saliva, GCF, or mouth rinse, or systemically from serum or plasma ([b] Pussinen et al. 2007). In serum, for example, concentrations of soluble CD14 (Jin and Darveau 2001; Jin et al. 2004), lipopolysaccharide-binding protein (LBP) (Ren et al. 2004), and toll-like receptors (TLRs) are elevated after exposure to periodontobacteria.

In order to resist the continuous exposure to microbes, the periodontium produces a wide range of pro-inflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs) that participate in the destruction of periodontal tissue. Following endotoxin activation, gingival epithelial and inflammatory cells start producing, for example, interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), IL-8, and intercellular adhesion molecules. Furthermore, the chemoattractant signals precipitate leukocytes and monocytes or macrophages to amplify inflammation in the infected periodontium (Uitto et al. 2003). The most common chemokines and cytokines suggested as markers of periodontitis in GCF are summarized in **Table 1**. Other markers of periodontitis include serum antibody levels against periodontopathogens (Papapanou et al. 2001; Pussinen et al. 2002; Dye et al. 2009; [b] Pussinen et al. 2011).

Lymphotoxin-α (LTA) cytokine, formerly known as TNF-β, is expressed by lymphocytes (Ware 2005) and has several proinflammatory activities in critical immunological processes (Vassalli 1992). The gene for LTA is located in the TNF gene cluster in the human major histocompatibility complex (MHC) class III region. Genetic polymorphisms in *LTA* associate with the risk of having periodontitis (Holla et al. 2001; Fassmann et al. 2003; Palikhe et al. 2008; Vasconcelos et al. 2012), but also with the susceptibility to coronary heart disease (Ozaki et al. 2002; Laxton et al. 2005).

Increased production of acute-phase proteins in the liver is activated by the proinflammatory cytokines originating from infected tissue. Periodontitis research has mainly focused on C-reactive protein (CRP), serum amyloid A (SAA), and fibrinogen ([b] Pussinen et al. 2007). These proteins defend the host from adjunct injuries by activating complement factors and participating in tissue regeneration.

MMPs are a family of catalytic enzymes that are capable of degrading extracellular matrix (ECM) proteins and are involved in multiple biological development and tissue repair processes, as well as pathological conditions such as periodontitis (Sorsa et al. 2006). They are secreted by the majority of cell types in the periodontium, and the expression is significantly increased in infection-induced periodontal inflammation. The main level of MMP activity control is regulation of the expression of genes coding for MMPs. The transcription is stimulated by cytokines, hormones, and growth factors such as IL-1β, TNF-α, estrogen, epidermal growth factor, and fibroblast growth factor (MacNaul et al. 1990; Ruhul Amin et al. 2003; Sorsa et al. 2006). In addition, the activity of MMPs is regulated by endogenous inhibitors, the tissue inhibitors of metalloproteinases (Uitto et al. 2003). Previous studies on the diagnostic utilization of MMPs have highlighted MMP-8, MMP-9, and MMP-13 as the main MMPs associated with periodontitis (**Table 1**) (Sorsa et al. 2014).

Table 1. The most common biomarkers of periodontitis in gingival crevicular fluid.

Molecule	Source	Function
CCL5	T-cells	Chemoattractant for inflammatory cells
IL-1β	Lymphocytes	Induces bone resorption and the production of other cytokines,
	Monocytes/macrophages	matrix-degrading enzymes, and prostaglandin E_2
	Endothelial cells	Inhibits bone formation
IL-6	Monocytes/macrophages	Induces the final maturation of B-cells
	Endothelial cells	Provokes antibody secretion
	Fibroblasts	
	T- and B-cells	
	Keratinocytes	
IL-8	Monocytes/macrophages	Facilitates neutrophil transit through the tissue
	Endothelial cells	
	Fibroblasts	
MMP-8	Polymorphonuclear	Collagenase
	leukocytes	Degrades interstitial collagen (type I, II, and III)
	Chondrocytes	Digests ECM and non-ECM molecules such as fibrinogen
	Fibroblasts	
	Epithelial cells	
	Monocytes/macrophages	
	Plasma cells	
MMP-9	Keratinocytes	Gelatinase
	Osteoclasts	Degrades denatured collagen and gelatin
	Neutrophils	
	Macrophages	
MMP-13	Chondrocytes	Collagenase
	Osteoblasts	Digests type II collagen ten times faster than types I and III
	Fibroblasts	Produced during bone development and in wound healing
	Plasma cells	Activates osteoclasts
TNF-α	Monocytes/macrophages	Induces synthesis of collagenase, IL-1, and prostaglandin E_2

CCL5, C-C chemokine ligand 5, also known as Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES); ECM, extracellular matrix; IL, interleukin; MMP, matrix metalloproteinase; TNF, tumor necrosis factor

Modified from Sorsa et al. 2006; Pussinen et al. 2007.

1.1.5. Genetic susceptibility to periodontitis

Clinical and radiological findings together with the patient's medical history form the basis for evaluating the severity of periodontitis. Similarly as in other chronic diseases, both genetic and environmental factors play a role in the development of chronic and aggressive periodontitis. Based on previous studies, multiple genes are likely to have some influence on the risk of or protection against periodontitis (Laine et al. 2012). The genes and their variants may affect the disease outcome via encoded proteins, or their expression, resulting in alterations in patient immunity and thereby in the disease outcome.

The supposed genetic background of aggressive periodontitis may be stronger than in chronic periodontitis. The heredity of aggressive periodontitis has attracted interest for decades (Saxen 1980), and the genetic trait has been shown in familial aggregation studies (Genco and Borgnakke 2013). The largest family study on aggressive periodontitis concluded that the disease is inherited as an autosomal-dominant trait in both Caucasian and African-American families (Marazita et al. 1994). Meng et al. reviewed the genetic studies on families suffering from aggressive periodontitis, showing that the frequency of affected siblings reached 40–50% in many families (Meng et al. 2011).

In relation to familial aggregation in chronic periodontitis, Shearer et al. reported that parents with poor periodontal health usually have descendants with similar problems (Shearer et al. 2011). However, there was no clear distinction between genetic and environmental factors. One twin study estimated that approximately 50% of the variance in chronic periodontitis is attributed to heritability (Michalowicz et al. 2000), but another study comparing the periodontal parameters of monozygotic and dizygotic twin pairs concluded that the role of genetics in chronic periodontitis may have been overestimated (Torres de Heens et al. 2010).

Case-control association studies suggest that single nucleotide polymorphisms (SNPs) in the genes for IL-1β, IL-1RN, IL-6, IL-10, CD14, vitamin D receptor, MMP-1, and TLR4 may be associated with chronic periodontitis, although most of the findings have lacked replication analyses in larger study cohorts (Laine et al. 2012). One specific area of interest in the human genome has been the human MHC region, which in addition to other infectious diseases, has been associated with periodontitis in smaller scale studies (Takeuchi et al. 1991; Nunes et al. 1994; Palikhe et al. 2008).

The purpose of the hypothesis-free genome-wide association (GWA) studies is to explore genetic variation associated with certain disease across the whole human genome. Again, studies on aggressive periodontitis have identified more distinct associations with the

disease compared to chronic periodontitis. For example, Schaefer et al. demonstrated aggressive periodontitis as the most severe form of periodontitis to be associated with the SNP located in glycosyltransferase gene *GLT6D1* (9q34.3) in German patients (Schaefer et al. 2010). The first genome-wide investigation of the periodontopathogen profile detected suggestive evidence of an association of 13 loci with periodontopathogen colonization (Divaris et al. 2012), but the findings did not reach statistical significance (threshold for significance $p < 5 \times 10^{-6}$). Neither did the two following GWA studies on chronic periodontitis find any significant associations (Divaris et al. 2013; Teumer et al. 2013). However, Divaris et al. suggested an association of six loci with different levels of chronic periodontitis, one of these located in the MHC region. The most recent GWA study detected 10 genetic loci associated with periodontitis phenotypes at the suggestive level of significance (Shaffer et al. 2014). A summary of GWA studies on chronic periodontitis is proivided in **Table 2**.

In addition to GWA studies, haplotype analysis may be an interesting approach for genetic mapping of periodontitis. A haplotype is a combination of SNP alleles along a region of a chromosome that are inherited together. To date, for example, haplotypes in the *IL-4* and *IL-6* genes have been associated with periodontitis (Holla et al. 2004; Nibali et al. 2008; Holla et al. 2008). In addition, MHC class II polymorphisms have been suggested to protect against aggressive periodontitis in an Iranian sample (Jazi et al. 2013).

Emerging interesting research areas include epigenetics and modern bioinformatics. Epigenetic variations are heritable differences in gene function without alterations in the nuclear DNA sequence, and they may have an important role in connecting the genotype and environment to an individual's phenotype, thereby providing new insights into susceptibility to periodontitis (Laine et al. 2012). Laine et al. succeeded in detecting periodontitis cases with a combination of the presence of *P. gingivalis*, *T. forsythia*, and *A. actinomycetemcomitans* species in gingival pocket sample cultivations, and SNPs *IL-1A* -889 and *TNF* -857 in new analysis based on bioinformatics tools (Laine et al. 2013). The model reached the sensitivity of 85% and specificity of 73%, and it may be valuable when considering the complex characteristics of periodontitis.

Table 2. Summary of genome-wide association studies showing suggestive evidence for an association with chronic periodontitis.

Study and phenotypes		SNP	Chr.	Closest gene	Beta / OR	SE / 95% CI	P-value
Divaris et al., 2013							
CDC-AAP[+]	Severe	rs12883458*	14	*NIN* (intronic)	1.89	1.48–2.41	$3.5x10^{-7}$
		rs2521634	7	*NPY* (not in gene)	1.47	1.25–1.73	$1.6x10^{-6}$
		rs11925054	3	*WNT5A* (not in gene)	1.69	1.37–2.10	$6.5x10^{-7}$
	Moderate	rs7762544	6	*NCR2* (not in gene)	1.41	1.24–1.60	$1.1x10^{-7}$
		rs3826782	19	*EMR1*(intronic)	2.00	1.48–2.70	$4.0x10^{-6}$
		rs12260727	10	*CELF2* (not in gene)	1.54	1.30–1.82	$6.0x10^{-7}$
Teumer et al., 2013							
20–81 years	Mean PAL	rs12497795	3	*EPHA3* (not in gene)	-0.08	0.02	$1.7x10^{-6}$
	PAL4Q3	rs7567687	2	*RAB6C* (not in gene)	0.76	0.68–0.85	$8.0x10^{-7}$
	CDC-AAP (mod. + sev.)	rs1953021	9	*C9orf150* (not in gene)	1.35	1.20–1.53	$1.2x10^{-6}$
	5-year change in mean PAL	rs2569991	3	*IQSEC1* (not in gene)	0.20	0.04	$1.3x10^{-6}$
20–60 years	Mean PAL	rs1875110	3	*ERC2* (intronic)	-0.13	0.03	$3.6x10^{-6}$
	PAL4Q3	rs1370967	5	*CAMK4* (not in gene)	2.21	1.61–3.02	$7.9x10^{-7}$
	CDC-AAP (mod. + sev.)	rs9822005	3	*MFSD1* (not in gene)	0.76	0.67–0.85	$3.7x10^{-6}$
	5-year change in mean PAL	rs11536940	20	*LBP* (intronic)	0.38	0.08	$2.2x10^{-6}$
	1000G mean PAL	rs9979250	21	*ETS2* (not in gene)	0.15	0.03	$4.1x10^{-7}$
	1000G CDC-AAP (mod. + sev.)	rs13237474	7	*FAM180A* (not in gene)	3.05	2.00–4.65	$2.4x10^{-7}$
Shaffer et al., 2014							
At least two sextants with PPD ≥5.5mm[#]		rs733048	4	*RAB28* (not in gene)	2.40	NA	$1.0x10^{-6}$
		rs10457525	6	*ARHGAP18* (not in gene)	2.33	NA	$3.5x10^{-6}$
		rs7749983	6	*ARHGAP18* (not in gene)	2.39	NA	$2.4x10^{-6}$
		rs10457526	6	*ARHGAP18* (not in gene)	2.26	NA	$6.0x10^{-6}$
		rs7816221	8	*HAS2* (not in gene)	2.12	NA	$9.2x10^{-6}$
		rs3870371	8	*HAS2* (not in gene)	2.15	NA	$5.6x10^{-6}$
		rs920455	8	*HAS2* (not in gene)	2.11	NA	$9.2x10^{-6}$
		rs12799172	11	*GVINP1* (not in gene)	2.12	NA	$5.1x10^{-6}$
		rs11659841	18	*CDH2* (not in gene)	2.48	NA	$9.4x10^{-6}$
		rs8094794	18	*FHOD3* (intronic)	2.17	NA	$5.9x10^{-6}$
		rs11713199	3	*OSBPL10* (intronic)	1.87	NA	$6.9x10^{-6}$
		rs12630254	3	*OSBPL10* (intronic)	1.90	NA	$6.7x10^{-6}$
		rs12630931	3	*OSBPL10* (intronic)	1.89	NA	$6.2x10^{-6}$
		rs733048	4	*RAB28* (not in gene)	1.95	NA	$4.4x10^{-6}$
		rs2297778	6	*AKAP12* (intronic)	2.32	NA	$9.7x10^{-6}$
		rs3783412*	14	*CDKL1* (intronic)	1.85	NA	$7.9x10^{-6}$
		rs12589327	14	*SEL1L* (not in gene)	2.13	NA	$6.6x10^{-6}$

*SNPs are located in the same chromosomal region (14q21) within a distance of 423 kb. [+]Severe and moderate chronic periodontitis classified according to the Centers for Disease Control and Prevention (CDC) in partnership with the American Academy of Periodontology (AAP); see also 1.1.6. [#] In addition, 14 subjects with self-reported "gum surgery". SNP, single nucleotide polymorphism; Chr, chromosome; Mean PAL, mean proximal attachment loss; PAL4Q3, first vs. third sex- and age-specific tertiles for the percentage of sites with proximal attachment loss ≥4 mm; Mod. + sev., moderate and severe chronic periodontitis; 1000G, analysis performed using the 1000 Genomes imputed variant set; NA, not available.

1.1.6. Definition of periodontitis

Epidemiological studies on periodontal diseases are complicated by the variety of definitions and methodologies used. The lack of globally accepted case definitions for periodontitis has been addressed by many authors (Albandar 2007; Page and Eke 2007; Savage et al. 2009). The most generally used clinical determinants for periodontitis have been CAL and PPD, and the disease has been categorized as mild, moderate, or severe (Page et al. 1997). However, Savage et al. revealed heterogeneity in the threshold for defining periodontitis in terms of CAL from 2 to ≥6 mm, and when PPD was used, from 3 to ≥6 mm (Savage et al. 2009). In addition, previous studies have used other parameters such as gingival inflammation, BOP, or radiographically defined alveolar bone loss for the definition of the disease.

The Group C consensus report of the 5th European Workshop in Periodontology (Tonetti et al. 2005) underlined that attachment loss should be the primary measure used in studies on the risk factors for periodontitis, and periodontitis cannot be determined by a single variable. Since CAL measures the accumulated past disease at a site, the report emphasized that in combination with attachment loss, additional measurement of the currently active disease status (BOP and/or PPD) is needed. The proposed criteria for the two-level periodontitis case definition by the European Federation of Periodontology to be used in epidemiological studies of risk factors are presented in **Table 3**. Elsewhere, the Centers for Disease Control and Prevention (CDC), in partnership with the American Academy of Periodontology (AAP), have focused on improving the surveillance of periodontal disease in the US adult population (Eke and Genco 2007). In 2007, they published their own case definitions for the population-based follow-up of periodontitis (Page and Eke 2007), which were updated in 2012 (Eke et al. 2012). The CDC-AAP case definitions for the surveillance of periodontitis are also presented in **Table 3**. Subsequently, Baelum and Lopez demonstrated that the case definitions presented by Tonetti & Claffey (2005) and by Page & Eke (2007) yielded similar results, which were also comparable to the results of simply identifying a case of periodontitis as a person having at least one site with both AL ≥4 mm and BOP (Baelum and Lopez 2012).

Table 3. Definition criteria for a "periodontitis case" for research purposes according to the AAP and EFP.

	EFP	CDC-AAP
Mild periodontitis	≥2 non-adjacent teeth with proximal AL ≥3 mm	≥2 interproximal sites with AL ≥3 mm and ≥2 interproximal sites with PD ≥4mm (not on same tooth), or one site with PD ≥5mm
Moderate to severe periodontitis	Proximal AL of ≥5mm in ≥30% of teeth	Moderate: ≥2 interproximal sites with AL ≥4mm (not on same tooth), or ≥2 interproximal sites with PD ≥5mm (not on same tooth) Severe: ≥2 interproximal sites with AL ≥6 (not on same tooth) and ≥1 interproximal site with PD ≥5 mm

EFP, European Federation of Periodontology (Group C consensus report of the 5th European Workshop in Periodontology; CDC-AAP, Centers for Disease Control and Prevention in partnership with the American Academy of Periodontology; AL, attachment loss; PD probing depth.

Modified from Tonetti and Claffey, 2005; Eke and Page, 2012.

1.1.7. Prevention and treatment of chronic periodontitis

Periodontitis is an insidious disease due to the lack of early explicit symptoms in affected patients. However, careful screening as a part of regular dental inspections helps to detect the early sings of periodontitis. Proper diagnosis, including risk assessment, is vital for accurate treatment. In Finland, the treatment of periodontitis is based on Current Care Guidelines (Könönen et al. 2010). The Current Care Guidelines for dentistry, generated by the Finnish Medical Society Duodecim and the Finnish Dental Society Apollonia, are independent, evidence-based guidelines for clinical practice. The basis of periodontal treatment is to eliminate the biofilm and plaque retentions in collaboration between the dentist, dental hygienist, and the patient.

The prevention of periodontitis demands intervention in the patient's oral hygiene, such as individual brushing and interdental cleaning instructions together with tobacco counseling. It has been shown that periodontitis is more common among subjects brushing their teeth less than twice a day (Knuuttila and Suominen-Taipale 2008), and smokers have a poor response to periodontal treatment compared to non-smokers (Paulander et al. 2004). The additional use of chlorhexidine may support the oral self-care and plaque removal of elderly and physically challenged people (al-Tannir and Goodman 1994).

Mechanical root debridement, i.e. scaling and root planing, in the removal of subgingival biofilm and calculus by hand and ultrasonic instruments retains its leading position in the cause-related nonsurgical treatment of chronic periodontitis (Sanz et al. 2012). Scaling and root planing exclusively are powerful in boosting periodontal attachment levels and decreasing inflammation. In cases with severe periodontitis, systemic antibiotics and surgical treatment may be used as adjuncts. Systematic maintenance care in addition to reinforcement of daily microbial plaque control practices is essential to achieve long-term success in periodontal therapy.

1.2. Lipoprotein metabolism

1.2.1. Lipoproteins

Lipoproteins are water-soluble complex aggregates of lipids and proteins that transport cholesterol and triglycerides through the vascular and extravascular body fluids to cells, which demand these compounds for anabolic and energy purposes. Lipoprotein particles are spherical-shaped with an amphiphilic outer layer of phospholipids (PL), free cholesterol (FC), and amphipathic apolipoproteins, and a hydrophobic core of lipids, mainly triglycerides (TG), and cholesteryl esters (CE) (Wasan and Cassidy 1998). The human plasma lipoproteins are categorized into five major classes according to their density, function, and protein composition: chylomicrons (CM), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). In addition, lipoprotein(a) [Lp(a)] is considered as a specific lipoprotein subclass (Kostner et al. 1981). The classes differ in their sizes and densities, and protein and lipid compositions. Several processes, including enzymatic reactions, the exchange of apolipoproteins, and transfer of lipids, constantly modify the size and lipid-protein contents of lipoproteins in the circulation (Gotto et al. 1986). The hydrated density of the lipoprotein particles is determined by their lipid-protein ratio: the denser lipoprotein contains more protein.

The largest lipoprotein particles, CMs, are mainly composed of TGs (86%), and their relative protein content is low. VLDLs are the second largest lipoprotein particles still primarily containing TGs, but also more proteins, phospholipids, and cholesterol than CMs. IDL particles represent the largest lipoprotein particles that contain more cholesterol than TGs, while approximately half of the mass of smaller LDL particles comprise cholesterol. Finally, HDLs are the smallest and densest lipoprotein particles, rich in protein and consisting of only 3% triglycerides. In plasma, HDL exists in discoidal and spherical forms, from which the spherical HDLs are divided into subclasses HDL_2 and HDL_3 according to the particle size. HDLs together with LDLs are the most abundant lipoproteins in the

circulation (Rader and Daugherty 2008). The major characteristics of human plasma lipoproteins are summarized in **Table 4**.

Table 4. Characteristics of human plasma lipoprotein fractions

	CM	VLDL	IDL	LDL	HDL_2	HDL_3
Density (g/ml)	<0.95	0.95–1.006	1.006–1.019	1.019–1.063	1.063–1.125	1.125–1.210
Diameter (nm)	75–1200	30–80	25–35	18–25	9–12	5–9
Composition (mass%)						
Prot*	1–2	8	19	22	40	55
PL	7	18	19	22	33	25
FC	2	7	9	8	5	4
TG	86	55	23	6	5	3
CE	3	12	29	42	17	13
Major apolipoprotein	apo B-48	apo B-100	apo B-100	apo B-100	apo A-I, apo A-II	apo A-I, apo A-II
Source	Intestine	Liver	VLDL	VLDL, IDL	Liver, intestine	Liver, intestine
Main function	Transport of exogenous TG and Chol	Transport of endogenous TG	Transport of endogenous TG	Transport of endogenous Chol	Reverse cholesterol transport	Reverse cholesterol transport

CM, chylomicrons; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; CE, cholesteryl ester; FC, free cholesterol; PL, phospholipids; Prot, protein; Chol, cholesterol. Specific lipoprotein class lipoprotein(a) is not included in this table.

*Does not include bound carbohydrate.

Modified from Gotto et al., 1986; Wasan and Cassidy, 1998.

1.2.2. Lipoprotein metabolism and lipid transport

This section describes the most important routes related in exogenous and endogenous lipid transport, and reverse cholesterol transport. In addition, an overview of lipoprotein metabolism is presented in **Figure 2**.

Cholesterol derives exogenously from dietary fats or endogenously via biosynthesis. The CM particles are produced in the intestine, where the postprandial fats are first emulsified by bile acids, hydrolyzed into free fatty acids (FFAs), monoacylglycerols, and non-esterified cholesterol, and further internalized by mucosal enterocytes. The resynthesized TGs and CEs are packed into CMs with PLs, FC, and apolipoproteins, and secreted into the lymphatic circulation (Green and Glickman 1981). In the endothelium of capillaries, the lipolytic activity of lipoprotein lipase (LPL) releases FFAs from the TGs of CMs. This results in CM remnants, which are subsequently delivered to the liver, internalized by the hepatic LDL-receptor related protein (Hussain et al. 1991), and used, for example, for bile acids synthesis. Albumin-bound FFAs are transported in the circulation to peripheral tissues for use as energy in muscles or for storage in adipose tissue (Havel 1997).

Most human cells are capable of synthesizing cholesterol, but the liver has a particularly important role in endogenous cholesterol synthesis (Dietschy et al. 1993). In addition, the liver produces several apolipoproteins used in lipoprotein assembly. Hepatic cholesterol and especially triglycerides synthesized by the hepatocytes are assembled into VLDL particles and secreted into the circulation. Similarly to CMs, LPL hydrolyzes TGs of the VLDL core into FFAs (Wasan et al. 2008), resulting in VLDL remnants or IDLs, which may be absorbed back into the liver or further remodeled to LDL by hepatic lipase. LDL particles are the major cholesterol-carrying lipoprotein particles in the circulation and provide cholesterol for peripheral tissues, for example for hormone synthesis and the assembly of cellular membranes. LDL is internalized in cells via receptor-mediated endocytosis and transported into lysosomes for degradation (Brown and Goldstein 1983). Most of the cells have strict feedback control of cholesterol uptake via the number of LDL receptors. The over-accumulation of LDL can be seen as elevated concentrations of plasma cholesterol, which is mainly controlled by the liver. Deviating from the other cells, macrophages may take up large amounts of cholesterol via scavenger receptors (SRs) leading to the formation of foam cells (Greaves and Gordon 2005), lipid accumulation, for example, in the arterial wall subendothelial space, and eventually the development of atherosclerosis (see 1.4.1). In macrophages, the modified lipoproteins are delivered to lysosomes, where CEs are hydrolyzed to FC and FFAs at an acidic pH. Acetyl-Co A acetyltransferase 1 (ACAT-1) catalyzes the esterification of FC into CE, while neutral cholesterol ester hydrolase (nCEH) acts in the opposite direction, hydrolyzing intracellular CE at a neutral pH (Sekiya et al. 2011). Neutral CEH action is the initial step of reverse cholesterol transport.

The accumulating cholesterol, mainly derived from LDL in peripheral tissues, is taken up by HDL particles and transported to the liver, where it is further disposed of via bile into the feces in a process called reverse cholesterol transport (Fielding and Fielding 1995). The lipid-poor apoA-I originating from synthesis by hepatocytes and intestinal cells interacts with ATP-binding cassette A1 (ABCA1), a transport protein on the surface of peripheral macrophages (Wang et al. 2001). Subsequently, lipid-poor apoA-I is enriched with FC to form nascent pre-β-HDLs, which is converted into α-HDL during cholesterol esterification reaction by lecithin-cholesterol acyltransferace (LCAT). The maturation into HDL occurs via the fusion of α-HDL particles. Both nascent and mature HDL particles induce cholesterol efflux from peripheral cells via ABCA1 or ABCG1 and ABCG4, respectively (Wang et al. 2004; Rader and Daugherty 2008), acting as preferred acceptors of cellular cholesterol. HDL particles are mainly cleared from the circulation via uptake in the liver. Alternatively, HDL-associated CEs are transferred into LDL and VLDL via the cholesterol ester transfer protein (CETP). The lipid content of HDL particles is actively modified by CETP and also by phospholipid transfer protein (PLTP) (Stein and Stein 2005). CETP transfers triglycerides from VLDL to HDL, and CEs in the opposite direction, while PLTP transports PLs from the lipolyzed VLDL and CM particles mainly to HDL. In addition, PLTP is able to convert HDL_3 into larger and smaller HDL particles (Jauhiainen et al. 1993).

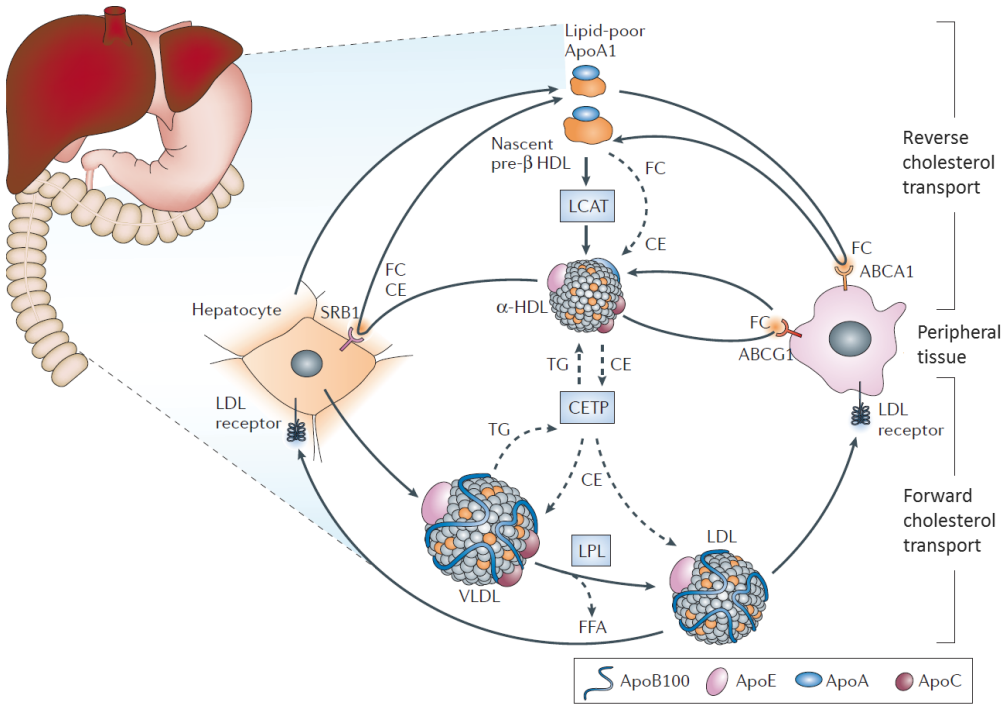

Figure 2. Lipoprotein metabolism. The transfer of lipids is represented by dashed lines, while intact lines represent lipoprotein pathways. (Wasan et al. 2008) Reprinted with permission from Nature Publishing Group.

1.3. Lipopolysaccharide

Lipopolysaccharide (LPS), often referred to as endotoxin, is a unique outer membrane structure and an important virulence factor of Gram-negative bacteria. It may originate from several sources, including infections, diet, and commensal microbiota. Gram-negative bacteria, e.g. *Escherichia coli*, *Chlamydia pneumoniae*, and periodontopathogens (see 1.1.3), are common pathogens colonizing the human gastrointestinal tract, including the oral cavity and the gut. The LPS molecule is essential for the viability of most Gram-negative bacteria, since it plays a crucial role in outer-membrane integrity as a permeability barrier, thereby protecting bacteria from toxic molecules. Bacteria may even fine-tune the structure of LPS to promote their survival. In the circulation, LPS interacts with several cell types, including epithelial cells, fibroblasts, macrophages, smooth muscle cells, T-cells, B-cells, and endothelial cells (Whitfield and Trent 2014).

1.3.1. Structure of LPS

LPS is a complex glycolipid composed of lipid and polysaccharide moieties joined by a covalent bond. The three structural regions of LPS are lipid A, a core oligosaccharide, and an O-specific side chain (O antigen). The biological activity of LPS is vitally dependent on the lipid A moiety, which is the most conserved part of the LPS and anchors the molecule to the bacterial outer membrane. It is a phosphorylated glucosamine disaccharide acylated with hydroxyl saturated fatty acids. Saturated fatty acids further 3-O-acylate the 3-hydroxyl groups of the fatty acids of lipid A (Raetz 1990). It has been shown that removal of the O-acylated saturated fatty acids or their substitution with unsaturated fatty acids leads to the disappearance of endotoxin activity (Munford and Hall 1986). The core oligosaccharide adheres directly to lipid A. The hypervariable O-side chain is a repetitive glycan polymer, which binds to the core oligosaccharide and forms the outermost part of the LPS (Manco et al. 2010). The repetitive units of the polymer may be linear or branched, and form homo- or heteropolymers (Raetz and Whitfield 2002). Each repeating unit represents diverse antigen properties, determining the serotype of the bacteria.

1.3.2. LPS-mediated signaling and the innate immune response

Through binding to the pathogen-sensing system, LPS induces the release of a large number of inflammatory cytokines, which play an important role in metabolic processes. The main elements of LPS-mediated signaling comprise lipoproteins, LPS-binding protein (LBP), cluster of differentiation 14 (CD14), an accessory protein (MD-2), and TLR4 (Bosshart and Heinzelmann 2007). These proteins act together to initiate a signaling pathway, which ultimately leads to the activation of nuclear factor-κB (NFκB) transcription

factor. In the circulation, LPS is transported by LBP, PLTP, and by lipoproteins to hepatocytes (Munford et al. 1981; Hailman et al. 1996). Approximately 80–96% of the LPS is bound to the lipoproteins via lipophilic lipid A (Levels et al. 2001; Harris et al. 2002), including all main lipoprotein classes (Levine et al. 1993). The process appears to be dependent not only on the content of phospholipids, but especially apolipoproteins such as apoA-I and apoE on the lipoprotein surface (Kitchens et al. 2003; Berbee et al. 2005). Lipoproteins receive LPS from LBP and PLTP. Under physiological conditions, LPS mainly associates with HDL, which contributes to its clearance via the liver and bile (Levine et al. 1993; Read et al. 1993). When the serum HDL is low, for example in sepsis patients, the majority of LPS is bound to VLDL (Levels et al. 2003). The TG-rich lipoprotein-LPS complex is rapidly eliminated by hepatocytes in order to reduce LPS-induced toxicity (Barcia and Harris 2005), or is internalized by macrophages (see 1.4.1) (Brown and Goldstein 1983). Therefore, the metabolic fate of LPS may be regulated by the lipoprotein profiles (Berbee et al. 2005).

LPS is bound to LBP, which transports the LPS molecules to soluble or membrane-bound CD14. For example, monocytes and neutrophils are activated via membrane-bound CD14, while endothelial cells are believed to respond to endotoxin exposure primarily through soluble CD14 (Stoll et al. 2004). PLTP is not able to transport LPS to CD14, and it is not therefore involved in this pathway of the immune response (Hailman et al. 1996). Alternatively, LBP may transport LPS to lipoproteins. Subsequently, the LPS-CD14 complex engages TLR4 via lipid A moiety (Chow et al. 1999). TLRs are needed in the downstream signaling pathway, since CD14 lacks a transmembrane domain. In addition, the secreted MD-2 binds to TLR-4 and LPS, thereby serving as an important factor of this receptor complex (Viriyakosol et al. 2001; Nagai et al. 2002). The activation of TLR4 leads to the recruitment of five additional adaptor molecules, including MyD88 and TRIF, which further trigger a cascade enabling NFκB to diffuse into the nucleus and activate the transcription of cytokines. TLR4 is the only TLR known to utilize all of these different adaptor proteins. (Lu et al. 2008) The most important proinflammatory cytokines produced by TLR4 activation are TNFα, IL1β, IL6, and chemokines (Stoll et al. 2004; Parker et al. 2007). In addition, TLR4 mediates the LPS response in vascular endothelial cells and in atherosclerotic plaques containing macrophages (Xu et al. 2001; Edfeldt et al. 2002).

1.3.3. LPS and periodontitis

Oral gingival epithelial cells act as a physical barrier against bacteria and play an important role in the host's innate defense (Andrian et al. 2006). In the progression of periodontitis, the composition of the biofilm changes from a predominance of Gram-positive bacteria to a majority of Gram-negative bacteria (Marsh 1994), and host cell invasion by periodontopathogens is regarded as a possible mechanism of chronic periodontitis

pathogenesis. For example, *Porphyromonas gingivalis* has the ability adhere to, invade, and replicate within epithelial cells (Kinane et al. 2008). Epithelial cells respond to bacterial challenge through pattern-recognition receptors, including TLRs, and activate the innate immunity system by expressing proinflammatory cytokines. Intact bacteria or bacterial products, including LPS, may enter the bloodstream through inflamed periodontal tissue and lymph vessels or via saliva to the gastrointestinal tract. It has been shown that transient bacteremia is a general occurrence after certain dental procedures, such as tooth extraction or periodontal examination (Olsen 2008). Indeed, bacteremia and endotoxemia are more common than previously thought, and are even induced by daily routines such as chewing or tooth brushing (Forner et al. 2006).

In the circulation, depending on the lipoprotein profiles, LPS may be complexed with the proatherogenic lipoproteins and internalized by macrophages, which may transform to foam cells (Brown and Goldstein 1983; Lakio et al. 2006). Hayashi et al. demonstrated that periodontitis patients have increased serum levels of soluble CD14 (Hayashi et al. 1999), which had earlier been correlated with increased mortality in bacteremia (Landmann et al. 1995). Via lipid A, 'red-complex' bacteria can impede the innate immune system by inhibiting the response of TLR4 to other microbes (Coats et al. 2005; Coats et al. 2007).

1.3.4. LPS and nutrition

In addition to the oral cavity, the gut is the other main source of LPS. Under physiological conditions, the intestinal epithelium defends itself from LPS translocation. However, LPS has a strong affinity for chylomicrons, and is able to easily cross the gastrointestinal mucosa (Ghoshal et al. 2009). The other suggested mechanisms for LPS translocation from the gut include uptake by intestinal enterocytes and microfold cells (Hathaway and Kraehenbuhl 2000), and alterations in the gene expression of host epithelial cells by Gram-negative bacteria (Hooper and Gordon 2001). A high-fat diet, obesity, diabetes, and non-alcohol fatty liver disease have been associated with increased permeability of the gastrointestinal mucosa, leading to metabolic endotoxemia (Neves et al. 2013).

In mice, chronic exposure of the host to LPS has been associated with the onset of insulin resistance, weight gain, and low-grade inflammation. A high-fat diet appears to favor the absorption of LPS across the intestinal barrier, and LPS appears to be a molecular link between a high-fat diet, the microbiota, and inflammation (Cani et al. 2007). Therefore, LPS may be identified as a novel factor triggering the onset of high-fat diet-induced obesity and type 2 diabetes (Manco 2009). Recently, Mani et al. demonstrated in pigs that serum LPS concentrations increased after a meal rich in saturated fatty acids (Mani et al. 2013). In a study on mice, a palm oil-based diet caused the most active transport of LPS to peripheral tissues via high LBP levels and low soluble CD14 levels, resulting in the

strongest inflammatory outcomes compared to milk fat, rapeseed, or sunflower oils (Laugerette et al. 2012). Since saturated fatty acids are able to affect the immune system and activate TLR4 (Fritsche 2006; Suganami et al. 2007), it is reasonable to assume that LPS acts synergistically with certain types of fatty acids, mainly saturated.

Besides animal studies investigating the relationship between dietary fat and LPS, studies in human subjects have shown that a high-fat and/or energy-rich diet may lead to low-grade endotoxemia (Erridge et al. 2007; Amar et al. 2008; Ghanim et al. 2009; Pendyala et al. 2012). Erridge et al. measured the plasma endotoxin concentration for 4 h after a high-fat meal in healthy men (Erridge et al. 2007). They discovered that endotoxin concentrations increased significantly after a high-fat meal alone or with cigarettes, but not after no meal or cigarettes alone. Moreover, Amar et al. observed a positive correlation between plasma LPS concentration and fat and energy intakes (Amar et al. 2008). Ghanim et al. demonstrated that a high-fat high-carbohydrate diet increased endotoxemia during 3 h after a meal compared with a meal rich in fruit and fiber (Ghanim et al. 2009). In addition to studies with healthy subjects, Harte et al. observed that a high-fat meal elevated circulating endotoxin irrespective of the metabolic state, but the postprandial elevation of endotoxin levels was stronger in groups with a high-metabolic risk, i.e. impaired glucose tolerance and type 2 diabetes mellitus, compared to non-obese controls (Harte et al. 2012). Dietary fats certainly appear to acutely increase the absorption of LPS via modification of the gut microbiota, increasing the amount of chylomicrons, and increasing the permeability of the gastrointestinal mucosa (Manco et al. 2010).

1.4. Cardiometabolic disorders

1.4.1. Atherosclerosis and cardiovascular diseases

Cardiovascular diseases (CVD) mainly caused by atherosclerosis lead to up to 16.7 million deaths every year, principally resulting from heart attacks and strokes (Dahlöf 2010). Atherosclerosis is described to be both a disorder of lipid metabolism and a chronic inflammatory disease of the large arteries (Ross 1999; Shibata and Glass 2009). It is a progressive and multifactorial disease having numerous risk factors contributing to the susceptibility to the disease. Established risk factors for atherosclerosis are divided into factors with a strong genetic component including family history of atherosclerotic disease, age, hypertension, male gender, increased concentrations of circulating LDL or VLDL cholesterol, reduced levels of circulating HDL cholesterol, elevated levels of lipoprotein(a), metabolic syndrome, diabetes, obesity, and depression, and environmental factors such as high-fat and high-sugar diet, smoking, low antioxidant levels, lack of exercise, and infectious agents (Lusis 2000). Risk factors act at several points on the

pathogenic pathway of atherosclerosis, and all of the genetic risk factors involve the function of several genes, with the exception of gender and the level of Lp(a). In addition, many inflammatory markers, such as CRP, may indicate systemic atherosclerotic alterations (Danesh et al. 1997).

Atherosclerosis is also a major cause of coronary heart disease (CHD). CHD, also known as ischemic heart disease or coronary artery disease (CAD), is the most common cause of premature death, particularly in industrialized countries. It was the leading cause of disability-adjusted life years worldwide in 2010, and stroke was ranked in third place in a recent review measuring the global burden of disease (Murray and Lopez 2013). CAD results from atherosclerotic, blood-flow reducing plaques, which narrow the arteries of the heart. Coronary angiography and other modern imaging techniques are utilized in the diagnosis of the disease.

Atherosclerosis is characterized by the formation of lipid-rich plaques within the artery walls. Persistently high levels of LDL, IDL, and VLDL particles lead to their accumulation in the vessel walls, which consist of three layers: the intima, media, and adventitia. The intima is the innermost layer with the endothelium on the luminal side, a basement membrane, and sub-endothelial connective tissue. The media consists of smooth muscle cells, and the adventitia is a layer of dense connective tissue, fibroblasts, and smooth muscle cells. (Libby et al. 2011) The formation of an atherosclerotic plaque begins when the proatherogenic lipoproteins invade the vessel endothelium, which has been damaged, for example, by hypertension, smoking, microbial infection, or hemodynamic forces in lesion-prone sites of the arteries. In the intima, the lipid and protein components of the LDL, IDL, VLDL, and Lp(a) particles become oxidized, which, in addition to other enzymatic modifications, leads to an inflammatory response (Hansson and Hermansson 2011). In addition, LPS complexed with the lipoproteins may further induce their oxidation. The host response to infection may further increase the oxidation of proatherogenic lipoproteins complexed with LPS (Memon et al. 2000). The endothelial production of adhesion molecules attracts monocytes, which migrate into the intima, differentiate into macrophages, and initiate cholesterol uptake by macrophage scavenger receptors such as SRA-1, CD36, and CD68 (Kunjathoor et al. 2002). When the blood LDL levels are persistently high, the imbalance between the influx and efflux of cholesterol induces the transformation of macrophages into foam cells loaded with CE-containing lipid droplets.

An initial atherosclerotic lesion is described as a fatty streak. During the disease progression, smooth muscle cells migrate to the intima from the medial layer of the vessel wall. In addition to macrophages, smooth muscle cells may also internalize CE and convert into foam cells. Macrophages may also became activated via TLRs (Moore et al. 2013), followed by an intracellular signaling cascade via NFκB transcription factor and the

secretion of inflammatory cytokines such as TNF-α and IL-6, chemokines, for example monocyte chemoattractant protein-1 (MCP-1), and proteases. Eventually, the recruited T-lymphocytes trigger a chronic inflammatory response. Smooth muscle cells proliferate in the intima, capture and retain proatherogenic lipoproteins, and form a sub-endothelial fibrous cap. Excess free cholesterol is cytotoxic, eventually leading to the death of foam cells, the release of cholesterol to the extracellular space, the accumulation of cholesterol in a necrotic core within the plaque, and even to the formation of cholesterol crystals. As a consequence, rupture of the fibrous cap involving the degradation of the extracellular matrix by MMPs may occur and further initiate coagulation of the blood due to the recruitment of platelets in the ruptured endothelium. Finally, the formation of a thrombus may lead to clinical events referred to as acute coronary syndrome (ACS), including acute myocardial infarction (AMI) and unstable angina pectoris. Inflammatory pathways are present in all stages of atherosclerosis, from the initiation to the final CVD event.

Endotoxemia has been shown to associate with the risk of incident CVD events (Wiedermann et al. 1999). Several previous seroepidemiological, histopathological, and microbiological studies, in addition to studies in animal models and clinical trials, have implicated the contribution of certain microorganisms, especially *C. pneumoniae*, *Helicobacter pylori*, cytomegalovirus, and periodontopathogens to the progression of atherosclerosis (Chatzidimitriou et al. 2012). Increased levels of antibodies against *C. pneumoniae* in stable CAD or AMI patients compared to healthy controls were already reported in the 1980s (Saikku et al. 1988), although some contradictory findings regarding *C. pneumonia* have also subsequently been published (Danesh et al. 2000; Ieven and Hoymans 2005). The presence of the intact pathogens or their nucleic acids within atherosclerotic lesions has been detected in several studies (Haraszthy et al. 2000; Ameriso et al. 2001; Kalayoglu et al. 2002; Kozarov et al. 2005). Various oral bacteria have been identified from the plaques since 1999 (Chiu 1999). In addition, other pathogens such as hepatitis C virus, herpes simplex virus, human immunodeficiency virus, and influenza virus have been detected from plaques (Ibrahim et al. 2005; Reszka et al. 2008; Chatzidimitriou et al. 2012). However, these findings *per se* do not prove a causal relationship with the atherosclerosis, since some pathogens may have been found stochastically.

Most of the pathogens associated with atherosclerosis are intracellular microbes causing long-lasting, persistent infections. Multiple infections together cause an infectious burden, which increases the risk of CVDs more than only a single pathogen (Epstein et al. 2000). Three different mechanisms for the pathogenic contribution to atherosclerosis have been suggested. First, pathogens may cause a local inflammatory reaction, promoting effects within the arterial vessel. Second, they may contribute to the progression of the disease via immune-mediated effects involving TLR stimulation and molecular mimicry, which

generates cross-reactive auto-antibodies. Third, genetic traits in the host response to infection and epigenetic factors may play a major role in the predisposition to CVDs. (Chatzidimitriou et al. 2012) **(Figure 3)**.

The structure and metabolism of lipoproteins change during the acute phase response (Khovidhunkit et al. 2004), characterized by nonspecific host responses against infection, inflammation, or injury. In general, plasma TG and VLDL levels increase and HDL levels decrease. In addition, VLDL and LDL particles are enriched with sphingolipids, especially ceramides, further increasing their uptake by macrophages (Memon et al. 1998), and the particle size of LDL diminishes, enhancing the oxidation (Tribble et al. 2001), whereas HDL is converted into a proinflammatory molecule called acute-phase HDL (Khovidhunkit et al. 2004). Regardless, the most evident reaction for the response is a dramatic increase in acute phase proteins, e.g. CRP, SAA, LBP, and fibrinogen. Serum LPS levels have been shown to correlate positively with CRP levels ([C] Pussinen et al. 2007; Lassenius et al. 2011). Generally, the alterations originating from the acute phase response protect the host from pathogens. However, if this state is prolonged, the changes in the composition and function of lipoproteins will promote atherogenesis.

1.4.2. Obesity, metabolic syndrome, and diabetes mellitus

Obesity is described as abnormal, excessive fat accumulation in both the subcutaneous and visceral space causing a risk to general health. It results from a long-term imbalance between the intake and consumption of energy interfered by environmental, genetic, and psychosocial factors (Kopelman 2000). Obesity interacts in several health problems both independently as well as in association with other diseases, including metabolic syndrome, type 2 diabetes mellitus, CHD, certain cancers, respiratory complications, and osteoarthritis (Kopelman and Albon 1997; Kopelman 2000). In addition to the level of food intake, the quality of the nutrition is relevant in weight gain and related disorders (Malik et al. 2006; Barclay et al. 2008; de Koning et al. 2011). There are several ways to measure overweight and obesity, including the body mass index (BMI), waist circumference or waist–hip ratio, and the body fat percentage. Most studies related to obesity have relied on the BMI-based classification by the World Health Organization (WHO) for adult overweight (≥25 kg/m^2) and obesity (≥30 kg/m^2) (World Health Organization 2004), which may be considered as the most useful population-level measure for the disorder.

Metabolic syndrome (MetS) is a group of metabolic disturbances, including abdominal obesity, hypertension, hyperglycemia, and dyslipidemia, which occur together more often than by chance alone and contribute to the development of CVD and diabetes (Lusis et al. 2008). The pathogenesis of MetS has multiple origins, but obesity, lifestyle, and genetic traits clearly interact in causing the syndrome. Over the past decade, several different

definitions for MetS have been introduced and a universally accepted definition is still lacking. The main difference between the definitions arises from the measure for abdominal obesity. The most frequently used definitions have been established by WHO (Alberti and Zimmet 1998), the European Group for the Study of Insulin Resistance (Balkau and Charles 1999), the US National Cholesterol Education Program Adult Treatment Panel III (Grundy et al. 2005), and the International Diabetes Federation (IDF) (Zimmet et al. 2005; Alberti et al. 2006). In 2009, the IDF, National Lung, Heart and Blood Institute (NHLBI), American Heart Association, World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity attempted to develop one global definition for the clinical criteria of MetS, resulting in a definition in which three simultaneous abnormal findings out of five would diagnose a person with the MetS: the population- and country-specific definition for elevated waist circumference (≥80 cm in females and ≥94 cm in males in Europids), fasting glucose ≥5.6 mmol/l or drug treatment for hyperglycemia, TG ≥1.7 mmol/l or drug treatment for hypertriglyceridemia, HDL-cholesterol <1.3 mmol/l in females and <1.0 mmol/l in males or drug treatment for reduced HDL-cholesterol, systolic blood pressure ≥130 mmHg and/or diastolic blood pressure ≥85 mmHg or antihypertensive drug treatment (Alberti et al. 2009). MetS is a significant risk factor for type 2 diabetes mellitus (T2DM) and CVD, and it increases mortality (Grundy 2005).

Diabetes mellitus (type 1 and type 2) originates from different chronic metabolic disorders characterized by disturbed glucose metabolism causing elevated circulating glucose concentrations. It results from abnormalities in insulin production or function. As in obesity, environmental, genetic predisposition, and psychosocial factors play a role in the susceptibility to the disease (Gerich 1998). The prevalence of diabetes is predicted to increase to epidemic levels in the coming 15 years (Chapple et al. 2013). The risk factors include for example impaired glucose tolerance, elevated fasting glucose, nutrition, physical inactivity, and a history of gestational diabetes. T2DM is the most common type of the disease and originates from a constant deterioration in the capacity of the pancreatic β-cells to secrete insulin. Consequently, defective insulin secretion is detected simultaneously with a decreased response of insulin-stimulated glucose uptake in the liver, muscle, and adipose tissues in a condition called insulin resistance, and the secreted insulin no longer compensates for the demands of the peripheral insulin. (Donath 2014)

1.5. Periodontitis and cardiometabolic disorders

1.5.1. Periodontitis and cardiovascular diseases

The first evidence for an association between oral infections and cardiovascular disease was already suggested in the late 1980s (Mattila et al. 1989; Syrjänen et al. 1989). Since then, numerous studies linking periodontitis and atherosclerosis have been published, most of them supporting the role of periodontitis in atherogenesis. Periodontitis and atherosclerosis are chronic, multifactorial diseases with a progressive nature, sharing various risk factors such as smoking, age, and diabetes mellitus, from which smoking is clearly a major risk factor for both diseases. Regardless, the diseases remain associated independently of known confounders (Lockhart et al. 2012). Both indirect mechanisms, including the effects of oral infection on systemic inflammation, and direct mechanisms, including transient bacteremia, endotoxemia, and proatherogenic changes in lipoprotein metabolism and vascular infection, have been suggested to promote the association. In addition, the effect of periodontal treatment on CVD risk factors has been investigated (Buhlin et al. 2009; Teeuw et al. 2014).

Inflammatory markers are important indicators of systemic inflammation. During a periodontal infection, proinflammatory cytokines and CRP are locally and systemically secreted from inflamed tissue (Page 1998). The inflammatory markers associated with periodontitis and CVD, e.g. elevated levels of CRP and IL-6, largely overlap (Loos et al. 2000; Lockhart et al. 2012). Another indirect mechanism linking periodontitis with atherosclerosis is molecular mimicry, which is caused by cross-reactive autoantibodies against periodontopathogens and heat shock proteins (Loos 2005; Lockhart et al. 2012). Access of oral bacteria or their products to the circulation is considered to be a key initiator of systemic events linking oral infections and CVD. In the circulation, infection also stimulates the secretion of cytokines, chemokines, and cellular adhesion molecules enhancing monocyte adhesion to the endothelium (Weill et al. 1995; Gerszten et al. 1998). For example, monocytes infected by *P. gingivalis* increased the expression of the TNF-α and IL-6 (Pollreisz et al. 2010). In addition, LPS induces lipid accumulation in macrophages, for example via down-regulation of SR-BI and ABCA1, thereby stimulating their transformation into foam cells (Funk et al. 1993; Khovidhunkit et al. 2001; Khovidhunkit et al. 2003; Lakio et al. 2006). Various seroepidemiological studies have associated an increased level of serum antibodies against periodontopathogens with prevalent CVD and an increased risk of future stroke, CHD, and MI (Pussinen et al. 2003; [a] Pussinen et al. 2004; Pussinen et al. 2005; Beck et al. 2005; [a] Pussinen et al. 2007; [c] Pussinen et al. 2007).

Intact periodontopathogens circulate in the bloodstream extracellularly or intracellularly within phagocytic cells, and are further deposited in atherosclerotic plaques. Moreover,

periodontopathogens may travel within platelets, resulting in platelet aggregates and the formation of thrombi (Iwai 2009), and they have been shown to invade endothelial cells (Deshpande et al. 1998; Dorn et al. 1999; Progulske-Fox et al. 1999). The survival and replication of pathogens within the cell is determined by the activation of autophagy and suppression of apoptosis. So far, periodontopathogen DNA has been found, for example, in carotid and coronary artery plaques, abdominal aortic aneurysmal walls and intraluminal thrombi, atherosclerotic vessel plaques, and cerebral aneurysms (Haraszthy et al. 2000; Okuda et al. 2001; Fiehn et al. 2005; Pyysalo et al. 2013; Pessi et al. 2013; Range et al. 2014), and other studies have even managed to demonstrate living bacteria within plaques (Kozarov et al. 2005; Rafferty et al. 2011). A potential mechanism linking periodontal infection to atherosclerosis and plaque rupture is presented in **Figure 3**.

Many case-control and animal studies have shown an association between unbalanced lipoprotein metabolism and periodontitis (Pussinen and Mattila 2004), which seems to be especially strong for apoB-100-containing lipoproteins (Griffiths and Barbour 2010). Periodontitis associates positively with total cholesterol, LDL, and TG concentrations, and negatively with HDL cholesterol concentrations (Lösche et al. 2000; Katz et al. 2002; Fentoglu et al. 2009). In a study by Ramirez-Tortosa et al., increased VLDL cholesterol associated with clinically diagnosed periodontitis (Ramirez-Tortosa et al. 2010). An increased number of small LDL particles was observed in a study on mice challenged with *A. actinomycetemcomitans* (Tuomainen et al. 2008). These particles are more susceptible to oxidation and more easily absorbed by macrophages, which was also shown in a treatment study ([c] Pussinen et al. 2004). Periodontitis may even disturb reverse cholesterol transport, since HDL of periodontitis patients appears to have a reduced capacity to remove cholesterol from macrophages ([b] Pussinen et al. 2004).

Most intervention studies have focused on the hypothesized positive effect of periodontal treatment on inflammation markers associated with CVD. Conservative periodontal therapy including scaling, root planing, and antibiotic treatment has been shown to reduce the levels of inflammatory mediators such as CRP, TNFα, and IL-6 (Iwamoto et al. 2003; D'Aiuto et al. 2004; Montebugnoli et al. 2005; Buhlin et al. 2009). In some other studies, periodontal treatment failed to alter to the levels of inflammatory mediators (Ide et al. 2003; Yamazaki et al. 2005), and in a meta-analysis, periodontal treatment failed to reduce systemic CRP levels (Ioannidou et al. 2006). However, a later systematic review concluded that there is modest evidence for the reduction of CRP levels by periodontal treatment (Paraskevas et al. 2008). Some studies have shown periodontal therapy to improve the lipoprotein profile, for example to reduce total cholesterol, LDL cholesterol, and oxidized LDL concentrations (Montebugnoli et al. 2005; Teeuw et al. 2014), but a study by Losche et al. detected no changes in serum lipid profiles after periodontal treatment (Losche et al. 2005). In addition, the benefits in periodontal health after six

months of treatment have been associated with improvement in endothelial function (Tonetti et al. 2007). The 2010 European Workshop in Periodontal Health and Cardiovascular Disease demonstrated that there is no inclusive evidence that preventive periodontal care or therapeutics will have an impact on cardiac health (Bouchard et al. 2010). Nevertheless, a very recent systematic review and meta-analysis concluded that periodontal treatment certainly reduces biomarkers of atherosclerotic disease and improves endothelial function, especially in those who are already suffering from CVD and/or diabetes (Teeuw et al. 2014).

Overall, there is a consensus that clinical periodontitis is associated with an increased risk of cardiovascular diseases through systemic inflammation as the etiopathogenic link (Lockhart et al. 2012; Tonetti et al. 2013; Dietrich et al. 2013). Moreover, causality in the association of periodontitis with CVD has been advocated (Belstrøm et al. 2012), although no studies to date have managed to prove a causative relationship (Lockhart et al. 2012). In 2013, Dietrich et al. systematically reviewed 12 strictly selected studies focusing on incident atherosclerotic cardiovascular disease including CHD, cerebrovascular disease, and peripheral arterial disease and periodontitis defined according to PPD or radiographically determined periodontitis (Dietrich et al. 2013). Apart from one study (Tuominen et al. 2003), all included studies detected a positive association between periodontitis and incident CVD independently of common CVD risk factors. Recently, the first prospective study on the relationship between the clinical and microbial periodontal profile and the progression of atherosclerosis was published by Desvarieux et al. (Desvarieux et al. 2013). They showed that an improvement in the periodontal status and a decrease in the amount of etiological periodontopathogen were associated with an improvement in carotid atherosclerosis (intima-media thickness as a surrogate marker for atherosclerosis) in a population-based sample of 420 participants followed for an average 3 years.

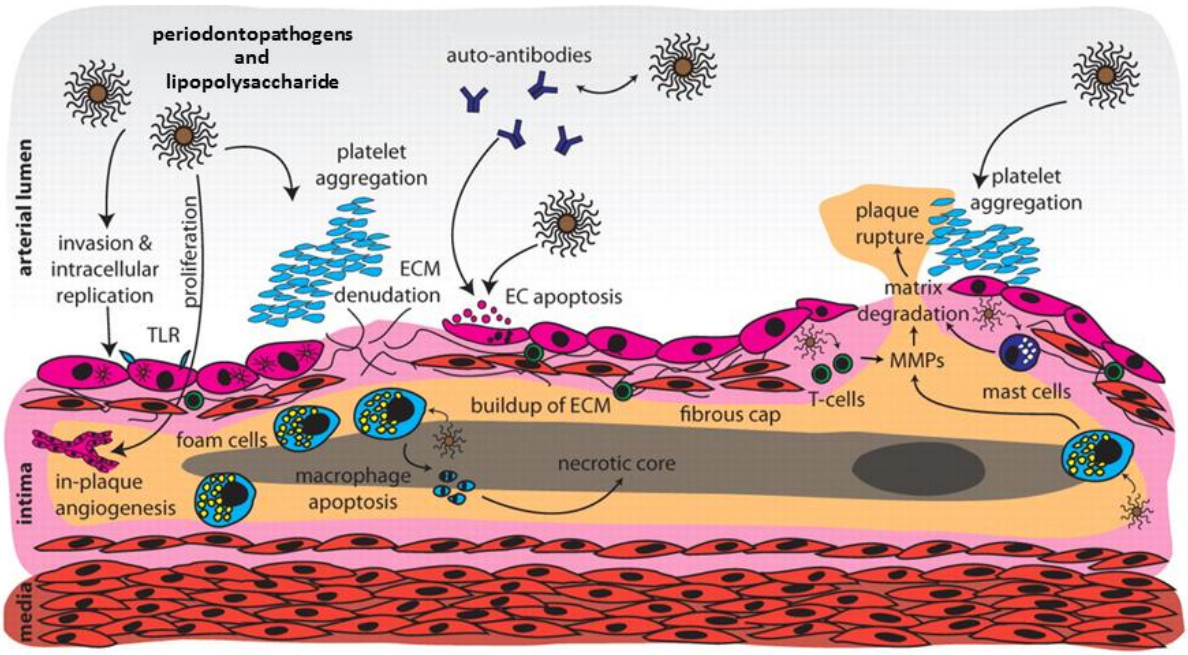

Figure 3. Potential mechanism linking the influence of periodontal infection on atherosclerosis and plaque rupture. Pathogen-mediated in-plaque angiogenesis is characteristic of plaque organization. Denudation of the fibrous cap and the release of its pro-thrombotic components occurs after endothelial cell (EC) apoptosis mediated by periodontal pathogens or auto-antibodies. Plaque rupture is induced by pathogen-mediated extracellular matrix (ECM) degradation by endothelial cells, macrophages, T-cells, and plasma cells. Modified from Kebschull et al. (2010) and reprinted with permission from SAGE Publications.

1.5.2. Periodontitis and obesity, MetS, and diabetes

Obesity has been regarded as a chronic condition with low-level systemic inflammation (Festa et al. 2001; Cancello and Clement 2006). Various epidemiological studies have demonstrated an association between periodontitis and elevated body weight (Genco et al. 2005; Linden et al. 2007; Ekuni et al. 2008; Haffajee and Socransky 2009). Furthermore, two recently conducted meta-analyses investigated the association between periodontitis and increased body weight (Chaffee and Weston 2010; Suvan et al. 2011). All the study data supported a positive association between periodontal disease and obesity, although the magnitude and causality remained unclear. Again, an important suggested mechanism linking the disorders was related to elevated systemic inflammation and the secretion of proinflammatory cytokines. For example, TNF-α levels of GCF and plasma were significantly correlated with BMI (Lundin et al. 2004; Khanna and Mali 2010), and hyperlipidemia has been shown to associate with higher values of periodontal parameters (Fentoglu et al. 2009). Recently, plasma levels of the inflammatory marker orosomucoid have been suggested as a potential biomarker of the association between periodontitis and obesity (Range et al. 2013). Endotoxemia has been reported to associate with energy and fat intake (Amar et al. 2008) and also with periodontitis ([c] Pussinen et al. 2007). Thus, elevated LPS concentrations in the circulation may also mediate the assumed connection. Interestingly, obese, but periodontally healthy individuals may suffer from overgrowth of

T. forsythia, which further predisposes them to periodontitis (Haffajee and Socransky 2009).

Increased insulin resistance is proposed as another linking mechanism for periodontitis, obesity, and MetS (Benguigui et al. 2010), since CAL increases together with insulin resistance (Genco et al. 2005). It has been shown that MetS may increase the risk of periodontitis (Shimazaki et al. 2007). It especially associates with the severe form of the disease (D'Aiuto et al. 2008), and seropositivity for *A. actinomycetemcomitans* has been shown to associate with MetS (Hyvärinen et al. 2014). In addition, a longitudinal study of 1023 subjects demonstrated that the presence of periodontal pockets was associated with the components of MetS during the observation period of 4 years (Morita et al. 2010). Oxidative stress and reactive oxygen species may partly explain this relationship, since both MetS and periodontitis have been shown to increase the serum levels of oxidative stress markers (Marchetti et al. 2012). From the components of MetS, it is presumable that obesity, diabetes mellitus, and low HDL levels affect periodontitis, since there is strong evidence of an independent association of these components with periodontal disease (Genco and Borgnakke 2013).

Diabetes mellitus is an established risk factor for periodontal disease. The relationship between the diseases appears to be bidirectional (Lalla and Papapanou 2011), with inflammation as an overarching feature. In diabetic patients, elevated levels of proinflammatory mediators contribute to the more severe form of periodontitis. Diabetes up-regulates inflammation in the periodontal tissues, and a hyperreactive response to periodontopathogens has been acknowledged to increase the severity of periodontitis in these patients (Genco and Borgnakke 2013). Periodontal inflammation may result in poor glycemic control in patients with diabetes, while those with good glycemic control may suffer little or no periodontitis. In addition, the patients with severe periodontitis in combination with diabetes suffer more often from cardiorenal mortality compared to patients with only diabetes (Genco and Borgnakke 2013). Diabetes increases the prevalence of periodontitis and affects the severity and progression of periodontal disease (Mealey et al. 2006; Taylor and Borgnakke 2008). Previous studies have demonstrated a higher prevalence and severity of periodontitis in both type 1 (Hodge et al. 2012) and T2DM patients (Fernandes et al. 2009) compared to subjects without diabetes. Furthermore, longitudinal studies have supported these findings, strengthening the conception of a bidirectional and causal relationship between the diseases (Demmer et al. 2008; Bandyopadhyay et al. 2010; Morita et al. 2012).

2. AIMS OF THE STUDY

The thesis study aimed to investigate whether LPS links periodontitis with cardiometabolic disorders. The genetics predisposing to periodontitis and the systemic effects of periodontitis-induced endotoxemia were investigated. The hypothesis was that triglyceride-enriched lipoproteins may have a role in periodontitis-induced atherosclerosis as proatherogenetic lipopolysaccharide carriers promoting systemic inflammation and foam cell formation, and that diet may affect the LPS-induced incidence of CHD. Therefore, the endotoxemia in patients with periodontitis and cardiometabolic disorders associating with periodontal disease were examined. In addition, the impact of periodontal treatment on plasma LPS activity and lipoprotein composition was investigated.

The specific aims of the thesis were:

1. To investigate whether genetic polymorphisms in the human MHC region are associated with periodontitis;
2. To determine the plasma lipoprotein compositions, including LPS activity, in periodontitis patients, and especially to investigate the proatherogenic properties of VLDL modified by endogenous LPS, as well as to examine the effect of periodontal treatment on LPS activity or its lipoprotein distribution;
3. To study the association of serum LPS activity with cardiometabolic disorders, and with the risk of incident CHD events, taking into account data on energy and macronutrient intake.

3. STUDY SUBJECTS AND METHODS

3.1. Study subjects and design

Table 5. Summary of the study populations.

		N (% of men)	Mean age (SD)	
Study I	Parogene 1	169 (72.2)	62.9 (9.9)	Random cohort of subjects with an indication for coronary angiography
	Parogene 2	339 (61.4)	63.7 (8.8)	
	Health 2000	1420 (46.1)	56.4 (8.1)	Population-based study on health and functional capability
Study II	Swedish periodontitis study	34 (55.9)	53.3 (8.1)	Periodontitis treatment study
Study III	Finnish periodontitis study	30 (53.3)	49.8 (7.4)	
Study IV	FINRISK97	2452 (48.7)	52.2 (10.1)	Population-based study on risk factors of chronic diseases

3.1.1. The Parogene study (I)

The large Corogene study was a prospective cohort study including 5294 patients who underwent coronary angiography at Helsinki University Central Hospital between June 2006 and March 2008 (Vaara et al. 2012). The aim of the Corogene study was to recognize coronary disease risk factors and the underlying genetics. Study I consisted of 508 subjects of the Parogene study (Buhlin et al. 2011), a random subsample of the Corogene study, including extensive clinical and radiographical oral health examinations. Among the Parogene study subjects, 123 had no significant CAD (<50% stenosis), 184 had stable CAD (≥50% stenosis), 169 had ACS, and 32 had ACS but not CAD. In study I, the ACS patients constituted the Parogene 1 population and the rest of the original Parogene patients formed the Parogene 2 population. The Parogene study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Helsinki University Central Hospital. All patients signed an informed consent form.

3.1.2. The Health 2000 Survey (I)

The Health 2000 Survey was an extensive nationwide study using a stratified two-stage cluster sample of 8028 adult citizens aged at least 30 years (Aromaa and Koskinen 2004; Knuuttila and Suominen-Taipale 2008; Heistaro 2008). The general aim of the survey was

to provide information on the health and functional capacity of the adult Finnish population. The sample, collected between 2000 and 2001 in Finland's five university hospital regions, included structured health interviews, clinical and radiographical oral health examinations, and blood sample collection. Study I was based on an age-limited subpopulation of 1420 adults (≥45 years) originally selected for a case-control GWAS of metabolic syndrome (Kristiansson et al. 2012). The cases had nondiabetic metabolic syndrome as defined by the IDF and the controls were age- and sex-matched healthy subjects. The Health 2000 Survey was carried out according to the Declaration of Helsinki and was approved by the Ethics Committee of the Helsinki University Central Hospital and the National Public Health Institute (KTL). The patients signed an informed written consent form.

3.1.3. Periodontitis treatment study in Sweden (II)

Study II comprised 34 Swedish patients (mean age 53 years) with periodontitis gravis et complicata (Nyman and Lindhe 2003). The exclusion criteria included a known history or medication for CVD, on-going infection, or any other chronic disease. The patients underwent an extensive periodontal examination including radiographs, and blood samples were obtained at baseline and on average six months after entering the study. The study was carried out in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the Karolinska Institutet. The patients signed an informed written consent form.

3.1.4. Periodontitis treatment study in Finland (III)

Study III included 30 Finnish patients (mean age 50 years) with periodontitis ([c] Pussinen et al. 2004). The patients had no diagnosed systemic diseases or infections within two months before enrolment in the study, and they had not received antibiotics during the six preceding months. The clinical oral health examinations were performed at baseline and three months after the periodontal treatment. In addition, blood samples were taken both before and three months after the periodontal treatment. The study was conducted in accordance with the Helsinki Declaration and the Ethics committee of the Institute of Dentistry at the University of Helsinki accepted the research plan. The patients signed an informed written consent form.

3.1.5. The FINRISK97 Study (IV)

The National FINRISK 1997 study was a population-based risk factor survey carried out in five geographical areas in Finland (Vartiainen et al. 2010), and comprised 8444 participants aged 25 to 74 years. Study IV was nutrition subsample (n = 2452) of the

FINRISK 1997 Study based on the National FINDIET 1997 Survey, which included clinical examination with height, weight, and blood pressure measurements, and blood samples in addition to a self-administered questionnaire. Information on macronutrient intake at baseline was collected from the 24-h diet recall interview. Obesity was defined as a BMI-based classification of adult overweight (≥25 kg/m^2) and obesity (≥30 kg/ m^2) according to the WHO (World Health Organization 2004), and MetS according to the IDF definition for Europids (Alberti et al. 2009). Prevalent diabetes and CVD events were defined as a doctor-diagnosed disease using the questionnaire, and the register data either as a purchase of related drugs or hospitalization with the disease. CHD events included subjects with a history of myocardial infarction, revascularizations, or percutaneous transluminal coronary angioplasty. In addition, a history of stroke (excluding subarachnoid hemorrhage) was included in prevalent CVD. Follow-up for incident CHD events was performed for 10 years with the use of record linkage of the FINRISK data with three data sources: 1) the National Hospital Discharge Register; 2) the National Causes of Death Register; and 3) the National Drug Reimbursement Register. The study was conducted in accordance with the Helsinki Declaration and the Ethics Committee of the National Public Health Institute (KTL) approved the study plan. The patients signed an informed written consent form.

3.2. Methods

The methods used in the thesis are listed in alphabetical order in the **Table 6**.

Table 6. Summary of the methods used in the thesis.

Method	Study	Section for details
Cell culture of human THP-1 monocytes	III	3.2.6.
ELISA		
ApoA-I	II	
IL-1β	II	
IL-6	II, III	
LTA	I	
MCP-1	III	
SAA	III	
TNF-α	II, III	
Enzymatic methods		
Glucose	IV	
GGT	IV	
HDL cholesterol	II, III, IV	
Phospholipids	II	
Total cholesterol	II, III, IV	
Triglycerides	II, III, IV	
Genotyping	I	3.2.2.
Gingival tissue biopsy	I	
Immunohistochemistry	I	3.2.3.
Immunoturbidimetry		
ApoA-I	II	
CRP	II, IV	
Isolation of lipoproteins	II, III	3.2.4.
Serum LPS activity (LAL assay)	II, III, IV	3.2.5.
Serum fibrinogen concentration (Clauss method)	III	
Periodontal examinations	I, II, III	3.2.1.
Periodontal treatment	I, II, III	3.2.1.
Protein concentration (Lowry method)	II, III	
Quantitative real-time PCR	III	3.2.7.
RNA isolation and cDNA synthesis	III	3.2.7.
Statistical analysis	I, II, III, IV	3.2.8.

3.2.1. Periodontal examination and treatment (I, II, III)

In the Parogene Study (I), a WHO manual periodontal probe was used for periodontal examination. PPD was measured from six sites of each tooth, and BOP and suppuration from four sites of the tooth, excluding wisdom teeth. The number of sites with a PPD of 4 to 5 mm and a PPD ≥6 mm were recorded. Digital panoramic radiographs were evaluated from both dentate and edentulous patients, and ABL, angular bone defects, and apical rarefactions were registered. ABL was classified into no bone loss, ABL in the cervical third of the root, ABL in the mid-third of the root, ABL in the apical third of the root, and total bone loss. It was calculated by selecting from each dentate sextant the tooth with the most severe loss of bone. For each patient, the mean value of these six measurements was calculated.

In the Health 2000 Survey (I), the examinations were also performed with a WHO manual periodontal probe. PPD was registered from four sites of each tooth, and the highest value of the tooth measured was recorded in categories PPD 4 to 5 mm and PPD ≥6mm. The wisdom teeth were excluded. BOP was determined by sextants (bleeding or not bleeding). ABL and angular bone defects were determined from the panoramic radiographs. ABL was classified similarly to the Parogene Study, and angular bone defects were categorized as follows: no vertical pocket, vertical pocket exceeding the middle third of the root, and vertical pocket exceeding to apical third of the root. The deepest measurement was registered. Study I did not include any periodontal treatment.

The Parogene Study and the Health 200 Survey populations were divided according to two different periodontitis definitions, advanced and severe periodontitis, as follows: i) advanced periodontitis included subjects with ABL in the middle third of the root to total bone support loss and two or more sites with a PPD of 4 to 5 mm or one or more sites with a PPD of ≥6 mm. The reference group included subjects with no ABL or ABL only in the cervical third of the root, but no PPD of ≥6 mm (healthy to mild periodontitis); ii) severe periodontitis comprised subjects with ABL in the middle third of the root to total bone support loss, a PPD of ≥6 mm at >3 sites, and a PPD of 4 to 5 mm at ≥10 sites. The reference group included subjects with no ABL, no PPD of ≥6 mm, and a PPD of 4 to 5 mm at <10 sites (healthy to gingivitis).

In study II, patients had been referred to a periodontist due to their severe periodontal disease. The comprehensive periodontal examination included radiographs. A WHO manual periodontal probe was used in the examinations and PPD was measured from six sites and visible dental plaque from four sites of each tooth. BOP was registered as the proportion of sites in the dentition that were bleeding. The patients had ≥7 sites with ≥6 mm CAL, horizontal loss of supporting tissue at least in the cervical third of the root with

BOP, furcation lesions in the multirooted teeth and/or angular bone defects with suppuration. The periodontal treatment included conventional mechanical periodontal therapy (scaling and root planing) and extractions of teeth due to periodontitis, caries lesions, endodontic or other reasons. In addition, the patients received oral hygiene instructions. The periodontal treatment finished when the patient was free from calculus and obtained good, self-contained oral hygiene. Patients were re-examined 3 months after the baseline examination. Standardized periodontal flap surgery was performed, if the patient still had a PPD of ≥6 mm. Again, the patients were re-examined 6 months after the baseline examination.

Study III patients were chosen from among those seeking periodontal treatment in Helsinki, Finland. They were selected for the study if they had at least 24 natural teeth, clinical (distance from the cementoenamel junction to the bottom of periodontal pocket exceeding 1 mm at approximal sites) and radiographical (distance from the cementoenamel junction to the alveolar bone margin exceeding 3 mm) attachment loss at more than 6 teeth, inflamed periodontal tissues, and they had had no periodontal treatment during the 6 preceding months. The clinical examination was performed at baseline and three months after the periodontal treatment. Radiographic examination at baseline included panorama radiographs with peri-apical radiographs if needed. The PPD, BOP, and suppuration were measured from six sites of each tooth. The periodontal treatment included traditional mechanical periodontal therapy as well as gingivoplasty and antibiotics according to the odontological needs of the patients (n = 7). Except for improved oral hygiene, the patients were not advised to change their daily habits, i.e. smoking or eating during the study.

3.2.2. Genotyping (I)

In Parogene 1, Parogene 2, and the Health 2000 Survey samples, DNA was isolated using standard salt precipitation protocols. The subjects in the Parogene 1 and the Health 2000 Survey were genotyped for SNPs with an Illumina 610K genotyping chip (Illumina HumanHap 610-Quad SNP array, San Diego, CA) at the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK). The subjects in the Parogene 2 were genotyped with Sequenom platform (iPlex MassARRAY, San Diego, CA) at the Institute for Molecular Medicine Finland (FIMM, Helsinki, Finland).

Altogether, 13,245 SNPs of the MHC region (6p21.3) with locations from 25749179 (rs932316) to 33473618 (rs6910741) were analyzed in the Parogene 1 sample. From these, 3,692 were genotyped SNPs. The quality control was conducted according to Anderson et al. (Anderson et al. 2010). In addition, before the genotype imputation, SNPs with a low call rate (<95%), low minor allele frequency, and low Hardy-Weinberg disequilibrium p-

value ($<1\times10^{-6}$) were excluded. The quality control was performed using PLINK software (Purcell et al. 2007). The cleaned dataset was imputed with MACH 1.16 using HapMap 2, release 22 CEU reference (http://hapmap.ncbi.nlm.nih.gov/downloads/phasing/2007-08_rel22/phased/). From the univariate analysis of the Parogene 1, 18 SNPs associating with periodontal parameters ($p \leq 0.001$) were selected for replication studies with the Parogene 2 and the Health 2000 Survey.

3.2.3. Histological analysis and immunohistochemistry (I)

Gingival tissue samples were obtained from healthy subjects (n = 2) and patients with chronic periodontitis (n = 2) via the collaboration with a Chilean research group. The samples were fixed in 4% buffered paraformaldehyde, embedded in paraffin, cut into 5-µm sections by a microtome, deparaffinized, and rehydrated. Antigen retrieval was performed by microwaving the samples in citric buffer (10 mM citric acid, pH 6, 0.05% Tween 20). The endogenous peroxidase activity was intercepted with a solution of 30% H_2O_2 in methanol, and the samples were subsequently blocked with goat normal serum and incubated with polyclonal antibody for LTA (HPA007729, 1:20 dilution, Sigma). Thereafter, the samples were incubated with biotinylated goat-anti-rabbit IgG as the secondary antibody (1:200 dilution, Vector Laboratories), and the antibody binding was visualized using an avidin-biotin-peroxidase complex and 3-amino-9-ethylcarbazole substrate (AEC, Sigma). Finally, the samples were counterstained with Mayer's hematoxylin and mounted with glycergel mounting medium (Dako).

3.2.4. Isolation of lipoproteins (II, III)

In study II, VLDL-IDL, LDL, HDL_2, HDL_3, and lipoprotein-deficient plasma (LPDP) were isolated from serum samples of all patients before and after periodontal treatment by sequential ultracentrifugation at an increasing density of solution (Havel et al. 1955). The density of the samples was adjusted with solid potassium bromide (KBr) to 1.006–1.019, 1.019–1.063, 1.063–1.12, 1.12–1.21, and >1.21 g/ml, for VLDL-IDL, LDL, HDL_2, HDL_3, and LPDP, respectively. The run time was two hours for VLDL-IDL, LDL, and HDL_2, and two and a half hours for HDL_3 with a Beckman Airfuge (running conditions: 160,000 g, +4 °C). HDL_3 and LPDP fractions were dialyzed against TBS (10 mM Tris-HCl, pH 7.3, containing 150 mM NaCl) before further determinations.

In study III, VLDL samples ($d < 1.006$ g/ml) of all patients before and after periodontal treatment were isolated by ultracentrifugation using a Beckman Optima Tabletop TL Ultracentrifuge with Beckman TLA 100.3 rotor at 424,000 g at +4 °C. The run time was two hours.

3.2.5. Serum LPS activity determinations (II, III, IV)

In studies II, III, and IV, the LPS activity of lipoprotein or serum samples was determined by a *Limulus* amebocyte lysate (LAL) assay coupled with a chromogenic substrate (HyCult Biotechnology B.V., Uden, the Netherlands). All samples were diluted 1:5 v/v in endotoxin-free water. After 45 minutes, the intensity of the substrate reaction generating a yellow color was spectophotometrically measured at 405 nm to reveal the amount of active clotting enzyme present in the sample, which correlates with the sample endotoxin concentration. Endotoxin activity is mainly reported as endotoxin units (EUs): an activity of 1 EU corresponds to 100 pg of *E. coli* lipopolysaccharide according to the WHO international standard (NIBSC code 94/580). The detection limit of the assay is 0.1 pg/ml and the interassay coefficient of variation was 9.2% (n = 75) ([b] Pussinen et al. 2011).

3.2.6. Cell culture (III)

Human THP-1 monocytes were purchased from the American Type Culture Collection (ATT, Manassas, VA, catalogue no. TIB-202). The cells were grown in complete RPMI 1640 medium, which contained 10% (v/v) fetal bovine serum, 10 mM HEPES, pH 7.4, 100 U/ml penicillin, and 100 µg/ml streptomycin, and maintained at + 37 °C under 5% CO_2 and 95% air. The monocytes were differentiated into macrophages with 30 ng/ml phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) for 24 h before the experiments. The differentiated macrophages were washed with phosphate-buffered saline (PBS), transferred to serum-free growth medium, and incubated for 18 h in the presence of VLDL (30 µg/ml as VLDL total protein) derived from periodontitis patients (n = 30) before and after periodontal treatment. The appropriate conditions for the cell experiments were optimized in a pilot study. Macrophages incubated in the absence of VLDL were used as control cells (n = 10). The growth media were collected after the incubation, centrifuged at 2000 rpm for 4 minutes, and supernatants were frozen at -70 °C. Finally, the macrophages were washed twice with PBS.

The lipid extract of the macrophages was collected by the addition of 0.5 ml of hexane/2-propanol (3:2, v/v) for 30 min at +4 °C, and dried under nitrogen. FC and CE were fractionated by high-performance thin layer chromatography and the spots quantified with an automatic plate scanner (CAMAG TLC). The remaining cellular residue was lysed in 0.3 N NaOH for 1 h at +4 °C. Furthermore, the protein content was measured by the Lowry method (Lowry et al. 1951).

3.2.7. cDNA synthesis and quantitative real-time PCR (III)

The extractions of total RNA from the macrophages were performed using an RNeasy® Mini Kit (Qiagen). Genomic DNA contamination was removed by a DNA-free™ Kit (Ambion). The concentrations of RNA were measured with an ND-100 (NanoDrop Technologies, Thermo Scientific). Synthesis of cDNA from total RNA was performed using ImProm-II™ Reverse Transcription system (Promega).

Primers for TNF-α, MCP-1, CD14, ABCA1, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were designed by Beacon Designire (Premier Biosoft International), and primers for IL-6, nCEH, ABCG1, SR-BI, ACAT1, CD36, and CD68 were designed by the National Center for Biotechnology Information Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and were tested for homology with unrelated sequences using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The relative mRNA expression levels were measured with quantitative real-time PCR (qPCR) using an Mx3005 Real-Time QPCR System (Stratagene). The expression of GAPDH (a widely used house-keeping gene) was used for data normalization.

For TNF-α, MCP-1, CD14, ABCA1, IL-6, and nCEH, duplicate samples were analyzed in 25 µl reaction mixture containing 2 µl of template DNA, 12.5 µl Brilliant® SYBR Green Master Mix (Stratagene), 0.375 µl ROX reference dye (final concentration 30 nM; Stratagene), optimized primer concentrations (described in publication III), and an appropriate volume of water (Sigma-Aldrich Co.) to adjust the reaction volume. The thermocycling program was as follows: initial denaturation at 95 °C for 1 min, 40 cycles of 1 min at 95 °C and 1 min at 60 °C, followed by the melting curve analysis: 1 min at 95 °C, a gradual decrease to 60 °C, 30 s at 60 °C, a gradual increase to 95 °C, and 30 s at 95 °C.

For ABCG1, SR-BI, ACAT1, CD36, and CD68, duplicate samples were analyzed in 20 µl reaction mixture containing 2 µl of template DNA, 10 µl Brilliant III Ultra Fast SYBR® Green QPCR Master Mix (Agilent Technologies), 0.3 µl ROX reference dye (Agilent Technologies), optimized primer concentrations (described in publication III), and an appropriate volume of water (Sigma-Aldrich Co.) to adjust the reaction volume. The thermocycling program was as follows: 95 °C for 3 min, 40 cycles of 10 s at 95 °C and 20 s at 60 °C, followed by melting curve analysis: 1 min at 95 °C, a gradual decrease to 60 °C, 30 s at 60 °C, a gradual increase to 95 °C, and 30 s at 95 °C.

Analysis of the results was performed using Mx3005 Real-Time QPCR System (Stratagene) software. The relative expression levels were calculated by a mathematical model for relative quantification in real-time reverse transcription PCR (Pfaffl 2001) and expressed as

the fold change compared to the control cells incubated in the absence of VLDL. The control cell level was set to one in the analysis.

3.2.8. Statistical analysis (I, II, III, IV)

In all studies, p-values <0.05 were considered to be statistically significant.

In study I, the Hardy–Weinberg equilibrium (HWE) was tested for each SNP with the χ^2-test, and the SNPs showing deviation from the HWE with a p-value of <0.01 or a minor allele frequency <1% were excluded. In the Parogene 1 population univariate analyses, 18 SNPs associated with periodontal parameters ($p \leq 0.001$), and these SNPs were selected for further analyses. The threshold for significance was set to 0.05/18 = 0.0028 (Bonferroni correction). Three different categories were used for smoking habits (never, former, and current). Logistic regression models were used to analyze the association between gene polymorphisms, risk haplotype, and periodontal parameters. These models were adjusted for age, sex, smoking, BMI, and diabetes, and the significance was assessed by the Wald test. In addition, in the analysis of the Health 2000 Survey population, the diagnosis of MetS and CVD, and a regional factor based on the University Hospital Areas in Finland were taken into account in the logistic models. PLINK software (Purcell et al. 2007) was used to analyze the associations in the Parogene 1 and 2 populations, and the Health 2000 Survey was analyzed using the ProbABEL package (Aulchenko et al. 2010). Linkage disequilibrium analysis was performed with Haploview software (version 3.32) (Barrett et al. 2005) and the haplotype reconstruction with FAMHAP (version 08/2008) (Herold and Becker 2009) and PHASE (version 2.1) (Stephens et al. 2001). The risk haplotype associating with periodontal parameters was constructed from SNPs with an r^2 value >0.9 according to the results from the Parogene 1 population. Conditional regression analysis was performed among the 6 associated SNPs in order to identify independent genetic markers. The analysis was not able to separate the effect of a single SNP due to the very high linkage disequilibrium. Therefore, further analyses were performed for the haplotype. Two-tailed Spearman correlation was used to analyze whether the serum LTA concentration had a correlation with other parameters, and the significances of differences between LTA concentrations and the LTA allotypes were analyzed by the non-parametric Mann–Whitney U Two-Independent-Samples test and the χ^2-test. The analyses were performed with IBM SPSS Statistics 20 Statistical Package for the Social Sciences.

In studies II and III, the non-parametric Wilcoxon signed-ranks test and Mann–Whitney U-test were used to test the statistical significance of differences between pre- and post-treatment samples, the subgroups, and the genders or smokers and non-smokers. Correlation analysis was performed by two-tailed Spearman correlation. For the analysis, we divided the patients into two groups according to medians of three clinical periodontal

variables: number of deepened periodontal pockets, BOP, and suppuration. In addition, to analyze the differences between patients with low and high systemic inflammation, the patients were divided into two groups according to the medians of CRP and fibrinogen concentrations. The analyses were performed with the Statistical Package for the Social Sciences (v.12.0. and v.15.0).

In study IV, the t-test or χ^2-test was used to analyze the differences between the subjects with and without cardiometabolic disorders. Serum TG, gamma-glutamyltransferase (GGT), and CRP values had skewed distributions and were logarithmically transformed before comparisons. Three categories were used for smoking habits (never, former, and current). Subjects were excluded from further analysis if the reported intake of total energy was less or greater than 3 x SD from the mean energy intake of the population (Missmer et al. 2002). A linear regression model was used to analyze the association between LPS and total energy or nutrient intake, first unadjusted, followed by a multivariate model including age, sex, education years, BMI, current smoking, and serum GGT, CRP, and cholesterol concentrations. The differences in LPS concentrations between lean, overweight, and obese subjects (BMI <25, 25–29.99, and ≥30 kg/m², respectively) were determined by the one-way analysis of variance. A logistic regression model was used to analyze the association of prevalent cardiometabolic disorders with the serum LPS activity.

In the models, the dependent variables were obesity, MetS, diabetes, or CHD, and the covariates included age, sex, education years, current smoking, hypertension (except the MetS model), cholesterol and CRP concentrations, and energy intake. Thereafter, the logistic regression models were further analyzed adjusting for protein, fat, and fiber intake instead of total energy. The subjects with prevalent CVD (n = 151) were excluded from the prospective analyses. The association of incident CHD events with the serum LPS activity was analyzed using Cox regression models adjusting for age, sex, years of education, current smoking, hypertension (except the MetS models), cholesterol, and CRP concentrations, and further for total energy or macronutrient intake. The analyses were performed with the IBM SPSS Statistics 21 Statistical Package for Social Sciences.

4. RESULTS

4.1. Genetics predisposing to periodontitis (I)

In total, the major histocompatibility class region comprised 18 SNPs, which were associated with periodontal parameters in the Parogene 1 population. From these, 10 SNPs remained significantly associated with periodontal parameters after adjustment for covariates. The strongest associations arose from the parameters BOP and PPD ≥6 mm with the genes *BAT1* (encoding HLA-B-associated transcript 1), *NFKBIL1* (encoding nuclear factor of κ light chain gene enhancer in B cells inhibitor-like 1), and *LTA* (lymphotoxin-α) locating in the MHC Class III region. A schematic diagram of the *BAT1-NFKBIL1-LTA* region is presented in **Figure 4**.

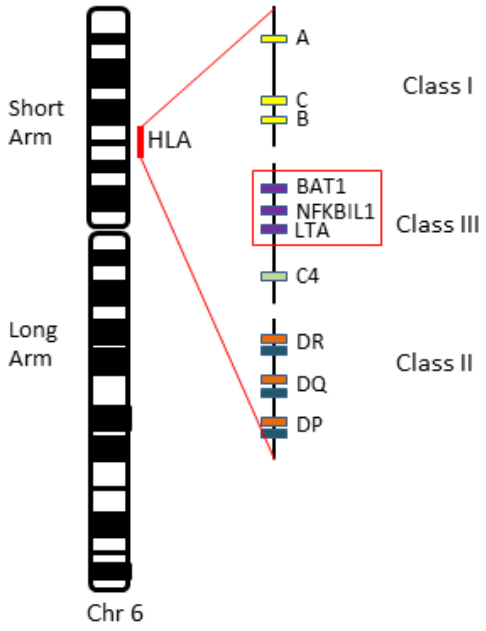

Figure 4. A schematic diagram of the *BAT1-NFKBIL1-LTA* region of major histocompatibility complex class III in chromosome 6. BAT1, encoding HLA-B-associated transcript 1; NFKBIL1, encoding nuclear factor of κ light chain gene enhancer in B cells inhibitor-like 1; LTA, lymphotoxin-α.

We tested the degree of pairwise linkage disequilibrium in 18 candidate SNPs associated with periodontal parameters by using r^2 statistics. The six SNPs, rs11796, rs3130059, rs2239527, rs2071591, rs909253, and rs1041981, with r^2 ≥0.92 constructed the risk haplotype. It was associated with BOP, PPD 4–5mm, and PPD ≥6 mm, but not with the number of teeth, ABL, or angular bone defects. In the Parogene 1 population, the strongest association emerged with BOP and PPD ≥6 mm, with an odds ratio (OR) 2.63

(95% CI 2.21–3.20, p = 0.00056) and 2.90 (2.37–3.52, p = 0.00042), respectively (**Table 7**). In the Parogene 2 and the Health 2000 Survey populations, the risk haplotype also significantly associated with the parameters BOP and PPD ≥6 mm. The ORs were 1.35 (1.10–1.72, p = 0.0097) and 1.49 (1.18–2.01, p = 0.0099), respectively, in the Parogene 2 population, and 1.38 (1.05–1.79, p = 0.0105) and 1.31 (1.06–1.61, p = 0.007), respectively, in the Health 2000 Survey population. The rest of the SNPs studied (n = 12) were not associated with the periodontal parameters in the replication populations.

Table 7. Associations between BOP(%) and PPD ≥6 mm and the SNPs comprising the risk haplotype in the Parogene 1 population.

			BOP (%)			PPD ≥6 mm		
Gene	**SNP**	**Risk Allele**	**Odds Ratio (95% CI)**	**p-value**	**Haplotype p-value**	**Odds Ratio (95% CI)**	**p-value**	**Haplotype p-value**
BAT1 intron	rs11796	A	2.5 (2.3–2.6)	0.0008		2.7 (2.3–3.2)	0.0008	
BAT1 intron	rs3130059	G	2.5 (2.3–2.6)	0.0008		2.7 (2.3–3.2)	0.0008	
BAT1 5' UTR	rs2239527	C	2.6 (2.2–3.1)	0.0003		2.9 (2.4–3.5)	0.0004	
NFKBIL1 intron	rs2071591	G	2.6 (2.2–3.1)	0.0003	0.00056	2.9 (2.4–3.5)	0.0004	0.00042
LTA intron	rs909253	A	2.5 (2.1–3.1)	0.0006		2.8 (2.4–3.4)	0.0007	
LTA exon [Thr] →[Asn]	rs1041981	C	2.5 (2.1–3.1)	0.0006		2.8 (2.4–3.4)	0.0007	

P-values obtained from the Wald statistic of the logistic regression model adjusting for age, sex, smoking, BMI, and diabetes. BOP, bleeding on probing; PPD, probing pocket depth; UTR, untranslated region; CI, confidence interval.

After analyzing the single periodontal parameters, we studied the association of the risk haplotype with advanced and severe periodontitis. Advanced periodontitis was significantly associated with the risk haplotype in the Parogene 1 population with an OR 1.69 (1.25–2.24, p = 0.041) compared to healthy and mild periodontitis. This finding was not replicated in the other populations. However, severe periodontitis was significantly associated with the risk haplotype in the Parogene 1, Parogene 2, and Health 2000 Survey populations compared to healthy and gingivitis subjects (**Figure 5**).

Since 5 of the 18 SNPs studied in the Parogene 1 population were located in the *LTA* gene and two of the SNPs belonged to the risk haplotype, serum LTA concentrations were measured in the Parogene 1 and 2 populations. The results showed that higher serum LTA concentrations were significantly associated with the risk alleles of the LTA SNPs rs909253 and rs1041981 when the homozygous subjects for these alleles were compared. The

median (interquartile range) LTA concentrations were as follows: AA 32.4 (13.1–68.8) pg/ml and GG 27.4 (15.3–164) pg/ml for rs909253 (p = 0.001), and CC 32.4 (13.1–86.8) pg/ml and AA 26.6 (13.8–273) pg/ml for rs1041981 (p = 0.001). In addition, LTA cytokine was localized in the inflamed gingival tissue in periodontitis, since connective tissue of the periodontal patient stained positive for LTA when compared to samples derived from the periodontally healthy subjects showing no LTA immunostaining. The immunostaining was seen in the surrounding stroma and on the surface of cells with apparent lymphocyte morphology.

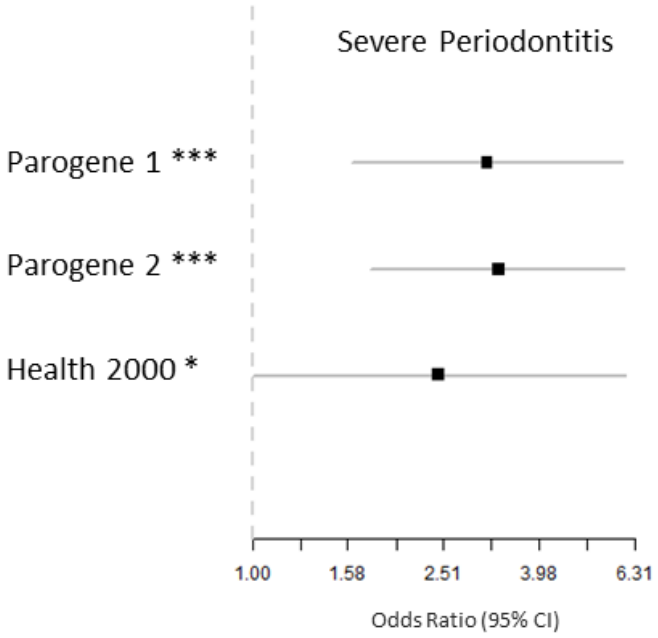

Figure 5. Association of the risk haplotype with severe periodontitis. Severe periodontitis was defined as follows: ABL in the middle third of the root to total bone support loss, a PPD of ≥6 mm at >3 sites, and a PPD of 4 to 5 mm at ≥10 sites. The reference group included healthy and gingivitis subjects with no ABL, no PPD of ≥6 mm, and a PPD of 4 to 5 mm at <10 sites. The logistic regression model was adjusted for age, sex, smoking, BMI, and diabetes in the Parogene, and additionally for regional factor, CVD, and metabolic syndrome in the Health 2000 Survey. The OR (▪) and the 95% confidence interval (CI). ***p < 0.001; *p < 0.05.

4.2. Endotoxemia in patients with periodontitis (II, III)

4.2.1. Plasma LPS activity and lipoprotein distribution in periodontitis patients before and after periodontal treatment (II)

In the treatment study conducted in Sweden, periodontal therapy was successful according to all registered clinical parameters. Among the plasma parameters, HDL cholesterol, apoA-1, and TG concentrations increased significantly after periodontal treatment, but changes in total and LDL cholesterol concentrations were not statistically significant. Furthermore, the inflammatory markers, CRP, TNF-α, IL-1β, and IL-6, remained unchanged after periodontal treatment.

The post-treatment plasma LPS activity was modestly, but significantly higher compared to pre-treatment values (55.7 ± 24.2 vs. 44.0 ± 17.0 EU/ml, p = 0.006). However, post-treatment plasma LPS levels decreased in 22 subjects (50.0 ± 18.8 to 41.4 ±17.1 EU/ml, p = 0.002). These patients had also higher pre-treatment HDL cholesterol (1.47 ± 0.47 vs. 1.24 ± 0.43 mmol/l, p = 0.044) and apoA-1 levels (1.68 ± 0.27 vs. 1.48 ± 0.32 g/l, p = 0.044), and lower CRP (1.16 ± 1.26 vs. 2.64 ± 3.11 mg/l, p = 0.028) and IL-6 concentrations (1.77 ± 1.61 vs. 3.44 ± 2.71 pg/ml, p = 0.011) compared to the 12 patients whose LPS levels were elevated. No clinical parameters, sex, age, or smoking habits explained the difference observed in LPS activities between the groups of 12 patients and 22 patients.

The LPS activity was distributed among the main lipoprotein classes as follows: VLDL-IDL 41.3 ± 12.1%, LPDP 25.0 ± 7.0%, HDL_3 13.1 ± 5.2%, LDL 11.5 ± 3.7%, and HDL_2 9.2 ± 2.8% (**Figure 6**). There were no significant differences in the LPS distribution between lipoprotein classes when comparing the pre-treatment and post-treatment values. Plasma LPS activity and VLDL-associated LPS activity correlated positively with depth of pathologically deepened periodontal pockets (r = 0.390; r = 0.345) and mobile teeth (r = 0.399; r = 0.484), and with CRP (r = 0.328; r = 0.367) and TG concentrations (r = 0.703, r = 0.427) ($p < 0.05$ in all cases). In addition, VLDL-LPS correlated positively with serum TNF-α concentration (r = 0.436, $p < 0.05$).

In addition, we determined the mass compositions of the lipoprotein classes before and after periodontal treatment. However, there were no significant changes in the compositions, except a minor decrease in the PL content of the VLDL-IDL fraction (29.0 ± 4.2% vs. 27.7 ± 3.6%, p = 0.049). The pre-treatment lipoprotein compositions are presented in **Table 8**. The cholesterol content of the VLDL-IDL fraction was surprisingly high at the cost of TG when compared to the expected composition in literature (**Table 4**).

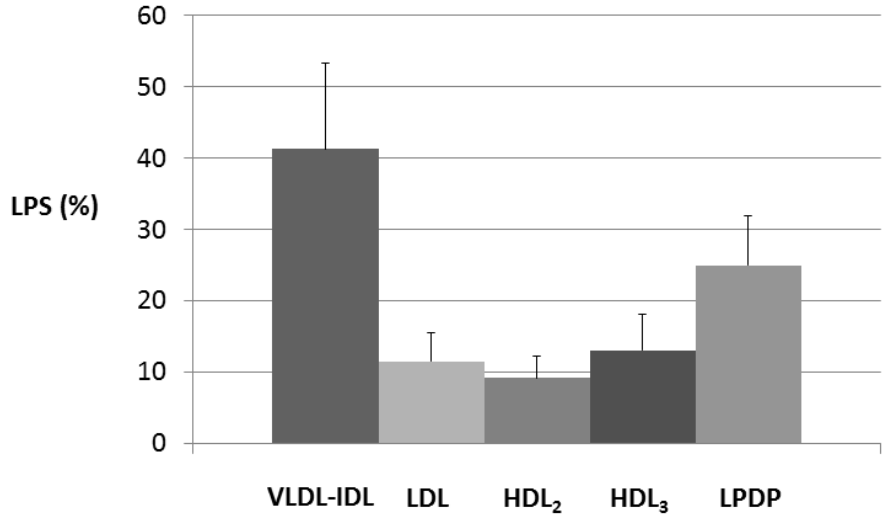

Figure 6. LPS distribution between lipoprotein classes before periodontal treatment. The lipoprotein fractions were isolated by sequential ultracentrifugation from 34 patients with periodontitis. LPS activity was measured using the *Limulus* amebocyte lysate assay. VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LPDP, lipoprotein-deficient plasma. Mean (SD) levels are shown.

Table 8. Lipoprotein compositions before periodontal treatment

	Mean mass composition (SD)			
	VLDL-IDL	LDL	HDL_2	HDL_3
Cholesterol (%)	40.9 (5.8)	36.1 (4.3)	21.7 (2.2)	17.0 (3.1)
Triglycerides (%)	16.0 (5.1)	9.1 (3.0)	5.2 (2.0)	3.5 (1.5)
Phospholipids (%)	29.0 (4.2)	40.0 (4.7)	33.4 (4.8)	34.4 (10.2)
Proteins (%)	14.0 (2.0)	14.9 (1.8)	39.7 (4.0)	45.1 (8.1)

*Wilcoxon signed-rank test

4.2.2. Proatherogenic properties of VLDL isolated from periodontitis patients before and after periodontal treatment (III)

The results from the study II prompted us to further investigate certain proatherogenic properties of VLDL-associated LPS. In the treatment study carried out in Finland, periodontal therapy appeared successful according to all clinical parameters registered. Compared to the baseline, the proportion of teeth with deepened periodontal pockets and the number of bleeding or suppurating periodontal pockets were significantly lower ($p < 0.001$) after periodontal treatment.

We determined the mass composition of the isolated VLDL before (**Figure 7**) and after periodontal treatment. There were no significant changes in the mean mass composition values. Additionally, the apoE content and LPS activities in the VLDL preparations and serum triglycerides, CRP, fibrinogen, and SAA did not differ significantly before vs. after treatment. However, serum HDL cholesterol, LDL cholesterol, and total cholesterol concentrations were higher after periodontal treatment.

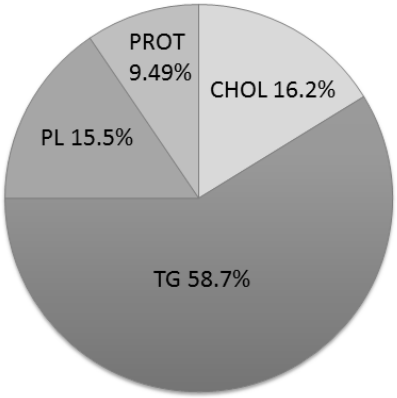

Figure 7. The mass compositions of VLDL before periodontal treatment. The mass composition data between the pre- and post-treatment VLDL particles did not differ significantly. In addition, there were no changes in apoE/total protein ratios or LPS activities in the pre- and post-treatment VLDL particles. PROT, total protein; CHOL, cholesterol; TG, triglyceride; PL, phospholipid. The mean mass percentages are presented.

The macrophages were incubated in the presence of pre- and post-treatment VLDL, and the cellular cholesterol content and expression of selected genes were measured. The contents of total, esterified, and free cholesterol in the macrophages incubated with VLDL preparations did not differ between pre- and post-treatment results. However, the uptake of VLDL measured as the cholesterol ester/total cholesterol ratio was nearly 2-fold higher ($p < 0.001$) compared to control LPS-free VLDL, and the uptake of pre-treatment VLDL correlated positively with the VLDL-associated LPS activity ($r = 0.436$, $p = 0.016$) and apoE content ($r = 0.374$, $p = 0.046$).

Periodontal treatment did not affect the potential of VLDL to alter the expression of the pro-inflammatory genes (TNF-α, MCP-1, IL-6, and CD14), or genes involved in cholesterol uptake and transport (nCEH, ABCA1, ABCG1, SR-B1, ACAT1, CD36, CD68). In addition, periodontal treatment did not affect cell culture media concentrations of the secreted pro-inflammatory cytokines (TNF-α, MPC-1, IL-6).

To further analyze whether the clinical symptoms had an effect on VLDL and macrophage inflammatory status, and on cholesterol metabolism, we divided the patients into two groups indicating moderate and severe periodontitis based on median levels of clinical symptoms. Before the treatment, the VLDL of the patients with more severe periodontitis induced higher mRNA expression of TNF-α ($p = 0.009$) and MCP-1 ($p = 0.0067$) than VLDL derived from the patients with moderate periodontitis **(Figure 8)**. In addition, the VLDL of patients with severe periodontitis with pus formation had higher LPS activity ($p = 0.017$)

and caused on average 18% higher cholesterol uptake (cholesterol ester/total cholesterol, p = 0.014) by the macrophages **(Figure 9)**.

The mRNA expression of TNF-α in macrophages incubated in the presence of pre-treatment VLDL correlated positively with other gene expression levels as follows: MCP-1 (r = 0.902, p < 0.001), CD14 (r = 0.887, p < 0.001), and IL-6 (r = 0.921, p < 0.001). There was a positive correlation between the TNF-α protein concentration in the culture media and mRNA expression of the 4 studied pro-inflammatory genes. In addition, a positive correlation of ABCA1 with nCEH and ABCG1 mRNA expression levels was demonstrated.

When macrophages were treated with pre-treatment VLDL preparations, the macrophage free cholesterol/total cholesterol ratio correlated significantly with the VLDL composition: VLDL-triglycerides (r = 0.487, p = 0.007) and VLDL-phospholipids (r = -0.368, p = 0.045).

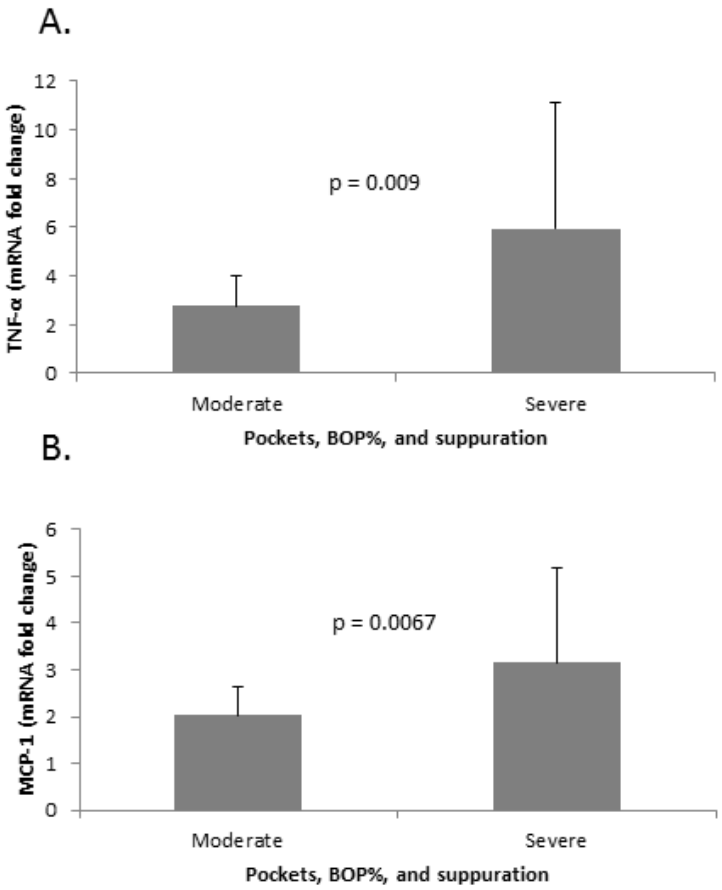

Figure 8. Macrophage gene expression levels and clinical symptoms of the periodontitis patients. Patients were divided into two groups (moderate and severe periodontitis) based on the median levels of clinical symptoms: periodontal pockets, bleeding on probing (BOP) and suppuration. The macrophage mean (SD) gene expression levels after incubation in the presence of VLDL preparations derived from the patients are shown for TNF-α (A) and MCP-1 (B).

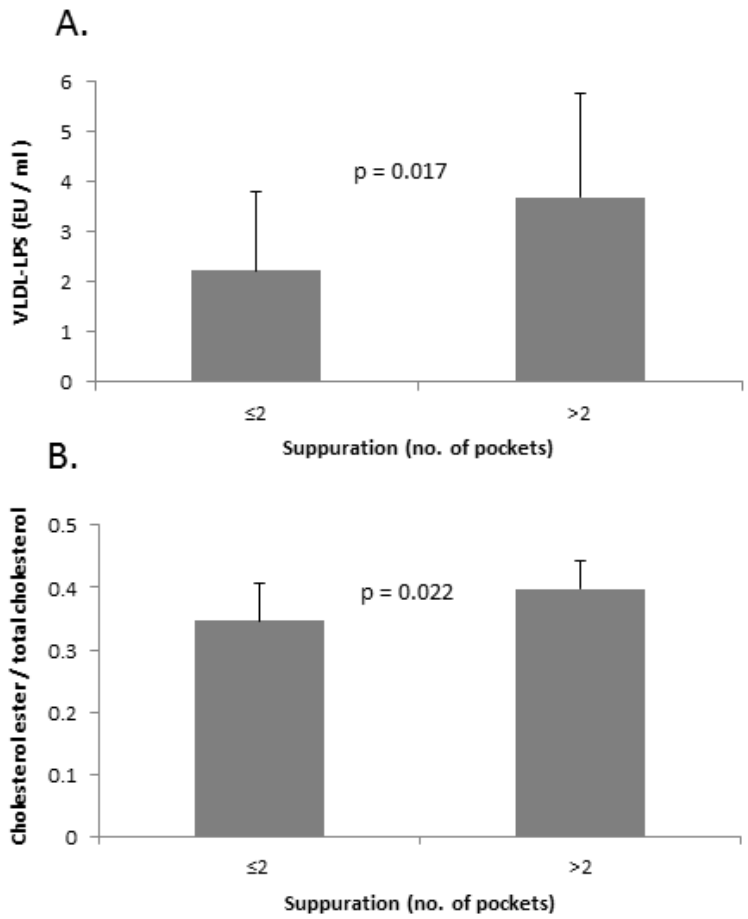

Figure 9. VLDL-associated LPS (A) and VLDL-induced cholesterol uptake by macrophages (B) in patients with different degree of inflammation in the periodontium. Suppuration is divided into two groups according to the median level observed in the population. Mean (SD) levels are shown.

4.3. Endotoxemia and nutrition in patients with cardiometabolic disorders (IV)

Prevalences of cardiometabolic disorders in the FINRISK 1997 nutrition cohort (n = 2452) at baseline are presented in **Figure 10**. During the follow-up of 10 years, altogether 137 incident CHD events appeared among the subjects with no history of CVD (n = 2301). At baseline, the majority of the established risk factors for cardiometabolic disorders differed significantly, as expected, when comparing the subjects with and without such disorders. The risk factors included age, sex, smoking, education years, serum total cholesterol, HDL cholesterol, TG, GGT, glucose, and CRP concentrations, and BMI, blood pressure, and prevalent MetS, diabetes, and CHD.

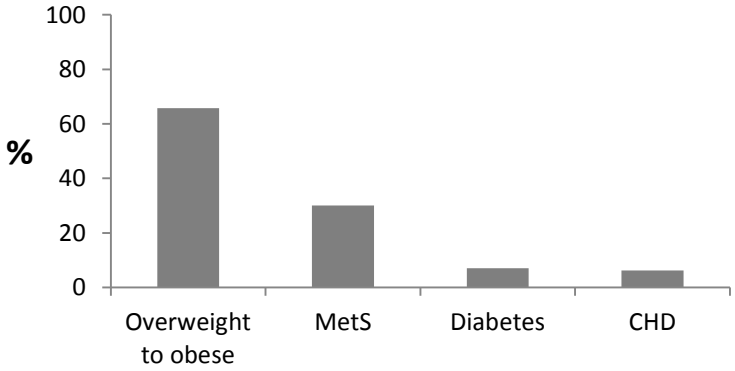

Figure 10. Percentage values of the cardiometabolic disorders in the FINRISK 1997 nutrition cohort (n = 2452). MetS, metabolic syndrome; CHD, coronary heart disease.

In a univariate linear regression models, there was no significant correlation between serum LPS activity and total energy, carbohydrate, or fat intake. In a multivariate model, however, LPS activity was directly associated with total energy intake, with unstandardized regression coefficients / 100 kcal (SE) of 0.84 (0.43, p = 0.050), and indirectly with the carbohydrate intake, -0.056 (0.02, p = 0.026). The associations were only seen in lean, healthy subjects, and not in subjects with obesity, MetS, diabetes, or CHD.

Subjects with prevalent obesity, MetS, and diabetes had significantly higher serum endotoxin activity compared to healthy subjects, but there was no statistically significant difference between those with and without prevalent CHD (**Table 9**). However, after 10 years of follow up, the mean (SD) serum endotoxin activity was significantly higher in subjects with CHD events compared to subjects without: 71.7 (39.1) vs. 62.8 (37.1) pg/ml (p = 0.006).

Table 9. Serum endotoxin activities in the prevalent cardiometabolic disorders.

	Obesity***		MetS***		Diabetes*		CHD	
	No	Yes	No	Yes	No	Yes	No	Yes
LPS activity (pg/ml)	54.2 (29.4)	68.5 (39.8)	56.6 (31.0)	79.6 (44.8)	63.2 (37.0)	68.9 (39.7)	63.4 (37.3)	68.0 (37.2)

Mean (SD) levels are shown. MetS, metabolic syndrome; CHD, coronary heart disease. ***p < 0.001; *p ≤ 0.05.

In the logistic regression models, high LPS activity was defined as follows: quartiles 2–4 compared to quartile 1 for obesity, Mets, and CHD, and pg/ml increase in the LPS activity for diabetes. High LPS activity significantly associated with prevalent obesity, MetS, diabetes, and CHD with the ORs (95% CI) of 1.49 (1.21–1.85, p < 0.001), 2.56 (1.97–3.32, p < 0.001), 1.01 (1.00–1.01, p = 0.032), and 1.94 (1.06–3.52, p = 0.031), respectively, when adjusted for total energy. The associations were independent of cardiometabolic risk factors, CRP, and total energy or macronutrient (protein, fat, and fiber) intake. **Table 10** presents the associations between risk factors and prevalent CHD (adjusted for total energy).

Table 10. Associations between risk factors and CHD

	Prevalent CHD[1]		**Incident CHD**[2]	
	OR (95%CI)	p-value	HR (95%CI)	p-value
Age (year)	1.12 (1.09–1.16)	<0.001	1.08 (1.06–1.10)	<0.001
Sex (male)	3.33 (1.94–5.71)	<0.001	2.80 (1.87–4.21)	<0.001
Education (year)	0.94 (0.87–1.0)	0.049	0.93 (0.87–0.98)	0.007
Current smoking	1.29 (0.75–2.21)	0.360	1.63 (1.11–2.41)	0.013
Hypertension	1.10 (0.67–1.81)	0.719	1.37 (0.91–2.06)	0.137
Chol (mmol/l)	0.63 (0.49–0.79)	<0.001	1.01 (0.86–1.20)	0.875
CRP (mg/l)	0.99 (0.95–1.03)	0.557	1.03 (1.01–1.06)	0.01
Energy (kcal)	1.00 (1.00–1.00)	0.371	1.00 (1.00–1.00)	0.196
High LPS*	**1.94 (1.06–3.52)**	**0.031**	**1.88 (1.13–3.12)**	**0.015**

[1]Logistic regression model [2]Cox regression model

*Q2–4 vs. Q1

During the follow-up of 10 years, altogether 137 incident CHD events appeared among the subjects with no history of CVD at baseline (n = 2301) (**Table 11**). **Figure 11** presents the Kaplan-Meier analysis for incident CHD events in LPS quartiles. Finally, discrimination of LPS activity for incident CHD events in the 10-year follow-up is presented in **Table 12**.

Table 11. Incident CHD events in LPS quartiles in the 10-year follow-up.

LPS quartiles	No. of subjects	No. of CHD events	Mean survival time (SEM)	HR (95% CI)*	p-value*
Q1	564	18	10.85 (0.037)	.	0.004
Q2	566	34	10.73 (0.050)	1.89 (1.06–3.35)	0.029
Q3	600	37	10.65 (0.065)	1.96 (1.11–3.43)	0.020
Q4	571	48	10.56 (0.029)	2.72 (1.58–4.67)	<0.001

*Cox regression model. LPS, lipopolysaccharide; CHD, coronary heart disease; SEM, standard error of mean; HR, hazard ratio; 95% CI, 95% confidence interval.

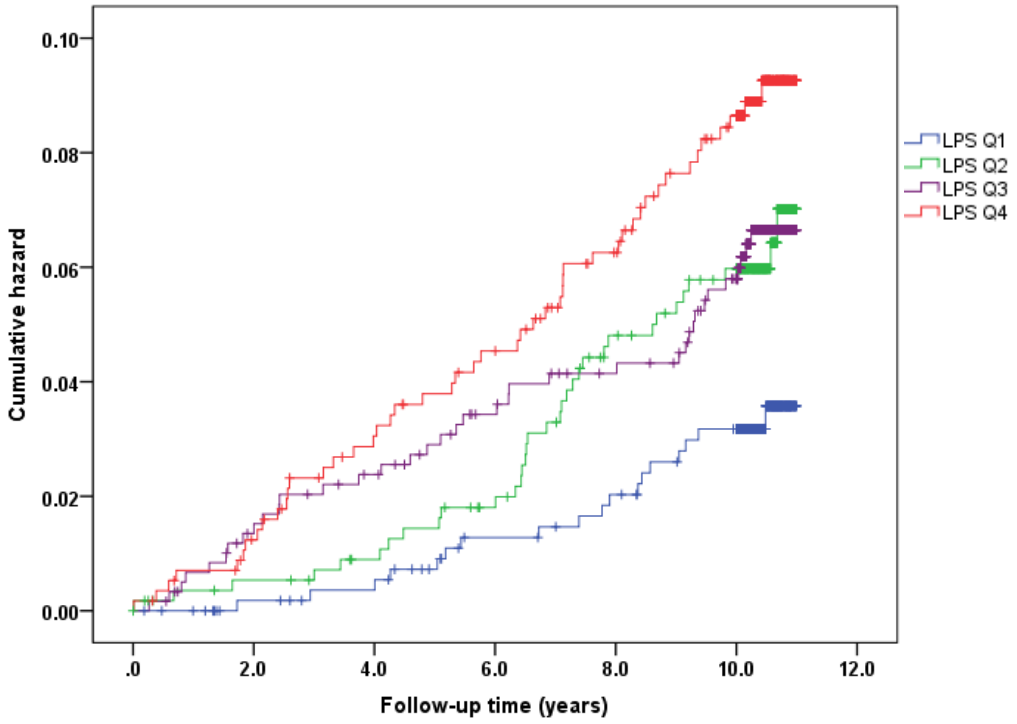

Figure 11. Kaplan-Meier analysis for incident CHD events in LPS quartiles (Q) in the 10-year follow-up. CHD events included subjects with myocardial infarction, coronary death, coronary bypass surgery, or percutaneous transluminal coronary angioplasty.

Table 12. Discrimination of LPS activity for incident CHD events in the 10-year follow-up.

	Reference risk score c-statistic	Extended risk score c-statistic	p-value
CHD events	0.565 ± 0.026	0.573 ± 0.026	0.996

Area under curve values ± standard errors are shown. The reference risk model included age, sex, education years, current smoking, cholesterol, and CRP concentrations, hypertension, and energy intake in the follow-up of ten years. The risk prediction model was extended with serum LPS activity (Q2–4 vs. Q1).

High LPS was significantly associated with incident CHD events in the multivariate analysis, with a hazard ratio of 1.88 (1.13–3.12, p = 0.015) (**Table 9**). The result did not change even if the model was adjusted for macronutrient intake instead of total energy, or further for prevalent obesity, MetS, or diabetes.

5. DISCUSSION

5.1. Genetic basis of periodontitis

Periodontitis is a complex inflammatory disease in which numerous causal factors simultaneously play a role (Laine et al. 2012). The chronic oral infection is mainly initiated by Gram-negative bacteria, but the subgingival biofilm alone is insufficient to induce the disease. Therefore, the destruction of the periodontal tissue is not caused by periodontopathogens *per se*, but rather by the inflammatory response of the host. Among the other environmental and risk factors for periodontal disease, genetic variations, mainly SNPs, have been suggested to associate with the susceptibility to periodontitis (Vaithilingam et al. 2014).

To date, only three GWA studies in relation to chronic periodontitis have been conducted, and the study results remain diffuse (**Table 2**). Hypothesis-free and unbiased analysis of GWA studies requires large sample sizes comprising thousands of cases to be able to confirm the risk variants involved (with the generally accepted significance level of $p < 5 \times 10^{-8}$) (Manolio 2010; Thompson et al. 2011), and rare variants strongly contributing to the genetic risk of a disease may not reach statistical significance at the genome-wide level. Therefore, haplotype analysis may turn out to be a promising approach in studies concerning the association between genetic polymorphism and periodontitis (Laine et al. 2012), as the findings of this thesis also demonstrate. Study I established a novel haplotype, which showed an association with the risk of periodontitis.

The risk haplotype was located on the MHC class III region and comprised six SNPs of the *BAT1*, *NFKBIL1*, and *LTA* genes with very high linkage disequilibrium ($r^2 \approx 1$). Since the Parogene 1 and 2 populations together formed the cohort and were strongly related to each other, the results were further replicated in an independent population, the Health 2000 Survey. The analysis revealed that the risk haplotype was common in the study population, with the lowest frequency of 42% in controls to the highest of 81% in cases. Interestingly, the *BAT1-NFKBIL1-LTA* region has been cited in studies concerning other inflammatory diseases, including CVD, suggesting that periodontitis and CVD may share genetic polymorphisms. It has been reported that there is at least a moderate genetic effect of the *BAT1-NFKBIL1-LTA* region in the modulation of risk for MI in Europeans and Japanese (Ozaki et al. 2002; Koch et al. 2007). In addition, a recent GWAS studying CAD found an association with 6p21 loci (Takeuchi et al. 2012), the same region where our haplotype located. Further investigations are still needed to improve understanding of the genetic factors interacting in periodontitis. In the future, the identification of novel polymorphisms associated with periodontitis may open new possibilities for diagnostics and prevention of the disease.

5.2. Periodontal parameters and definition of periodontitis

The symptoms of periodontitis include gingival bleeding, increased periodontal pocket depth, and destruction of periodontal ligaments and alveolar bone. Finally, the disease may lead to the loss of teeth. In study I, periodontitis was not among the original selection criteria for the study populations, since Parogene 1 and 2 included middle-aged or older symptomatic patients who underwent coronary angiography, and the Health 2000 Survey was a population-based sample with an age limitation of ≥ 45 years. Therefore, the idea was first to study the registered clinical signs of periodontitis: the number of teeth, BOP, PPD, ABL, and angular bone defects. The results indicated that the risk haplotype had the strongest association with the periodontal parameters PPD ≥ 6 mm and BOP. Although the investigation of single periodontal parameters may reduce the misclassification of subjects, the association of genetic polymorphisms with periodontitis itself as a disease was further examined. In particular, it was of interest to compare the healthy subjects with those having severe periodontitis, the so-called periodontitis extremes. The risk haplotype was significantly associated with severe periodontitis compared to healthy subjects and subjects with gingivitis.

PPD and BOP have been considered indicators of the current pathology of periodontitis, whereas CAL is a cumulative measure of the loss of support caused by the aggregate effects of pathogenic factors such as periodontitis and trauma (Lockhart et al. 2012). In addition, the number of teeth and ABL describe the history of periodontal disease. The definition of periodontitis is usually a combination of pocket formation and ABL, as also in the analysis in study I for advanced and severe periodontitis. The aim was to combine the different states of periodontitis by using the parameters PPD and ABL. However, together with PPD, CAL is regarded as a major parameter in the definition of a “periodontitis case” according to current European and American criteria (**Table 3**). It was not possible to determine CAL in study I, since the cementoenamel junction of the teeth was not registered, and therefore our definitions for periodontitis are not directly based on those given by the EFP or the CDC-AAP. Neither was the cementoenamel junction of the teeth registered in studies II or III.

In dentistry, different definitions and classifications typically have a minor significance for the clinical practitioner, since the screening of cases in need of treatment is the first priority. This thesis research comprised study populations with a distinct periodontal status and general health. In study I, the periodontal status of patients with cardiologic problems and subjects from the population-based study was investigated. Patients in studies II and III were generally healthy, but diagnosed to have periodontitis and to be in need of periodontal treatment. Therefore, the criteria for a “periodontitis case” in these studies were more exclusive than the criteria for advanced periodontitis in study I. In

study II, patients were included if they had at least seven sites with at least 6 mm loss of clinical attachment. In addition to the periodontal parameters examined in study I, visible plaque, teeth with furcation lesions, and number of mobile teeth were investigated in study II before and after periodontal treatment. Furthermore, in study III the patients had to have more than 6 teeth with clinical and radiographical attachment loss and inflamed periodontal tissues. The lack of an ideal classification system for a "periodontitis case" remains the major limiting factor in determining and comparing results across periodontal research.

5.3. Proinflammatory mediators

The innate immune system is dependent on pathogen recognition by certain receptors, such as TLRs, to initiate an immune response. The NFκB family of transcription factors is an important activator of genes associated with innate and adaptive immunity, inflammatory responses and the development and maintenance of the immune system (Bonizzi and Karin 2004). The NFκB pathway has been recognized as responsible for mediating many functions of proinflammatory cytokines, which may be a multidirectional link among periodontitis and cardiometabolic disorders. In the circulation, LPS binds to a pathogen-sensing system and induces the release of various inflammatory cytokines, which play an important role in different metabolic processes. In the present thesis, cytokines LTA, TNF-α, MCP-1, and IL-6 were especially investigated.

LTA expression by lymphocytes (T, B, and natural killer cells) is critical to various inflammatory processes (Vassalli 1992). In study I, 5 of the 18 significant SNPs associating with periodontal parameters were located in the *LTA* gene. Interestingly, *LTA* polymorphism has also been associated with the susceptibility to periodontitis in some previous studies (Holla et al. 2001; Fassmann et al. 2003; Palikhe et al. 2008; Vasconcelos et al. 2012). Our risk haplotype further comprised two *LTA* SNPs, from which the SNP rs1041981 is exonic with a threonine-to-asparagine change (Posch et al. 2003). The other SNP, rs909253, has previously been associated with periodontitis (Fassmann et al. 2003; Vasconcelos et al. 2012), but also with other chronic diseases and MI (Ozaki et al. 2002). Indeed, previous studies indicate that LTA and the immune system are also involved in lipid homeostasis, and the role of LTA in lipid-associated diseases such as MI and atherosclerosis has been investigated. Schreyer et al. demonstrated that a loss of LTA but not TNF-α reduced atherosclerosis in mice (Schreyer et al. 2002). In addition, LTA may induce the expression of various adhesion molecules and other genes in human endothelial cells, thereby promoting atherosclerosis (Suna et al. 2008). A recent meta-analysis concluded that SNP rs1041981 may be associated with susceptibility to MI, whereas SNP rs909253 may increase susceptibility to MI only in Asians (Li et al. 2014). In study I, all Parogene patients but only a few among the Health 2000 Survey subjects

suffered from cardiologic problems. Therefore, the association of the risk haplotype with CVD was not reasonable to investigate in our study.

In study I, *LTA* SNPs were associated with the presence of periodontitis, and the homozygous subjects had higher serum LTA concentrations. This clearly prompted us to consider whether LTA is expressed in periodontitis-affected gingival tissue. Since gingival biopsy samples were not available from Parogene 1 and 2 patients, from whom serum samples were available, a limited number of samples available from random periodontitis patients and healthy controls were decided to use. LTA was localized in the inflamed gingiva, suggesting that *LTA* polymorphisms may contribute to the regulation of inflammatory processes in periodontitis. LTA has the potential to be a new target molecule in periodontal research.

LTA polymorphisms have been shown to influence TNF-α production, and the effect is induced by the stimulus of LPS or Gram-negative bacteria (Temple et al. 2003). In study II, VLDL-bound LPS activity from periodontitis patients correlated positively with serum TNF-α concentrations, and in study III, the VLDL derived from periodontitis patients induced the gene expression and protein secretion of TNF-α and MCP-1 in macrophages, thereby potentiating their inflammatory activation. Although in study I no association of serum LTA with LPS levels was detected, periodontitis as a source of LPS may induce elevated serum LTA levels in the risk allele carriers. Thereby, LTA may play a significant role in mediating the systemic effects of periodontitis.

In addition, the risk haplotype comprised regions of the *BAT1* and *NFKBIL1* genes. BAT1 has been suggested to be a negative regulator of inflammation via the down-regulation of acute phase cytokine production (Allcock et al. 2001). NFKBIL1 has also been considered as an inhibitor of NFκB, and a lower expression of NFKBIL1 has been detected in periodontal ligament fibroblasts of periodontitis patients compared to control subjects (Scheres et al. 2011). This may suggest that NFκB is more easily activated in periodontitis patients. Furthermore, polymorphisms in the *NFKBIL1* gene have also been linked to other inflammatory diseases, e.g. rheumatoid arthritis (Lin et al. 2006). The role of *BAT1* and *NFKBIL1* polymorphisms in periodontitis needs to be further examined.

5.4. The effects of periodontitis-induced endotoxemia on lipoproteins

Periodontal pathogens and their LPS may continuously have access to the systemic circulation via inflamed periodontal pockets or saliva through gastrointestinal tract, thereby inducing systemic inflammation as LPS responses (Wahaidi et al. 2011; Erridge 2011). In addition, periodontitis may induce proatherogenic alterations in lipoproteins (Pussinen and Mattila 2004), since up to 80–96% of plasma LPS is carried by lipoprotein

particles (Levels et al. 2001; Harris et al. 2002). The key finding of study II was that the majority of serum LPS activity (41%) was associated with the pro-atherogenic VLDL-IDL fraction in periodontitis patients. In addition, this fraction was highly enriched with cholesterol. Plasma or VLDL-IDL-associated LPS correlated positively with the severity of periodontitis measured as the depth of deepened periodontal pockets, as well as CRP, and the plasma TG concentration, and negatively with plasma HDL cholesterol. There was a significant increase in plasma TG levels after periodontal treatment, which is in concordance with the modest increase in LPS. This may also be due to variance in fasting time.

Indeed, the association between periodontitis and disturbances in lipoprotein metabolism appears to be most distinct for apoB-100 containing lipoproteins (Griffiths and Barbour 2010). In addition to increased LDL cholesterol and TG levels, and decreased HDL cholesterol concentrations (Buhlin et al. 2003; [b] Pussinen et al. 2004; [c] Pussinen et al. 2004), periodontitis associates with an elevated VLDL cholesterol concentration (Ramirez-Tortosa et al. 2010). Although VLDL has been shown to be independently associated with the presence and progression of atherosclerosis (Tatami et al. 1981; Rutledge et al. 2000), it has rarely been characterized in periodontitis patients. In study II, we fractionated all the main lipoprotein classes. The isolated VLDL-IDL fraction was highly enriched with cholesterol, which supports a previous study on VLDL (Ramirez-Tortosa et al. 2010). It has been suggested that LDL and VLDL lipoproteins may replace HDL as the dominant LPS carrier during acute infection and inflammation, since HDL levels are low (Kitchens et al. 2003). After demonstrating in study II that a substantial portion of LPS activity is found in the VLDL fraction, the next target was to examine the effect of periodontal treatment on the proatherogenic properties of VLDL in study III.

In study III, it was observed that the VLDL of patients with severe periodontitis induced higher mRNA expression and protein secretion of TNF-α and MCP-1 in macrophages when compared with the VLDL derived from the patients with moderate periodontitis. LPS challenge induces the expression of inflammatory genes in macrophages, and it has been shown that VLDL potentiates LPS-induced TNF-α expression in macrophages (Stollenwerk et al. 2005). This inflammatory activation of macrophages was also uniquely evident in study III with endogenous VLDL-LPS. In addition, the VLDL of patients with severe periodontitis with pus formation contained more LPS and caused higher cholesterol uptake by macrophages. Small-sized VLDL may be taken up by macrophages to produce foam cells (Gianturco et al. 1982), while large VLDL with a diameter exceeding about 75 nm are excluded from the intima (Nordestgaard and Zilversmit 1988). As a limitation, there was no opportunity to measure VLDL particle sizes in studies II and III. However, VLDL cholesterol uptake by macrophages was associated with the LPS content of the VLDL particles, and the macrophage cholesterol content correlated positively with the VLDL

triglycerides and negatively with the phospholipids in study III. This suggests that the cholesterol uptake process in macrophages favored triglyceride-rich particles with high LPS activity. Thus, LPS associated with VLDL particles may promote macrophage inflammatory gene expression, foam cell formation, and eventually atherogenesis.

5.5. Local and systemic effects of periodontal treatment

In both treatment studies (studies II and III), the periodontal treatment was successful according to all periodontal parameters registered. Although local healing of the periodontium was accomplished, the systemic inflammation status and the lipoprotein profile failed to improve. In study II, the mean LPS activity increased after periodontal treatment due to 12 patients, while in 22 patients the LPS activity in fact decreased. This may be more or less explained by the preferable systemic inflammation status of the 22 patients: they had higher HDL cholesterol and lower CRP and IL-6 serum levels before treatment compared to the other 12 patients. In study III, periodontal treatment affected neither the VLDL composition, including its LPS content, nor cholesterol uptake by macrophages. The association of LPS with VLDL may further increase the proatherogenic function of circulating LPS due to the chronic nature of periodontitis. In addition, the results indicate that these proatherogenic properties associated with VLDL metabolism are dependent on the severity of the disease and are refractory to periodontal treatment.

A recent systematic review and meta-analysis concluded that CRP, IL-6, TNF-α, and total cholesterol significantly decreased, and HDL cholesterol increased after periodontal treatment (Teeuw et al. 2014). In particular, patients with co-morbidities, such as CVD and diabetes, benefitted most from periodontal therapy. In studies II and III, the inflammation-associated markers did not decrease after periodontal treatment (3-month and 6-month follow-up). This reflects the complexity and persistence of the disease, whereby a short follow-up time may complicate the observation of major improvements in the systemic inflammatory status. However, the study populations were relatively small (n = 34 and n = 30) to detect statistically significant changes in the inflammation status before and after treatment. In addition, patients expressed notable heterogeneity, including their age and diverse characteristics of the periodontal disease, which evidently affected the differences observed in treatment responses. Particularly in study III, the standard deviations and ranges were wide when analyzing the whole study population. Therefore, some of the results are based on subgroup analyses, i.e. subjects with moderate and severe periodontitis and subjects with low and high systemic inflammation markers. Studies with longer follow-up times may be needed to further analyze the systemic effects of periodontal treatment. Moreover, the role of genetics in the treatment outcome remains to be investigated.

5.6. Endotoxemia, cardiometabolic disorders, and diet

A number of epidemiological studies have demonstrated that periodontitis is a risk factor for many chronic systemic diseases and conditions, such as CVD, obesity, MetS, and T2DM (Lalla and Papapanou 2011; Lockhart et al. 2012; Genco and Borgnakke 2013). The most likely sources of circulating LPS levels are chronic infections by Gram-negative microbes, such as periodontal pathogens. In addition to the dissemination of periodontopathogens and LPS from the inflamed periodontium to the systemic circulation, they may be transported to the gut via saliva. It has been shown in mice that periodontopathogens may associate with elevated blood endotoxin levels by decreasing the gene expression of tight junction proteins in the gut epithelium (Arimatsu et al. 2014). Furthermore, LPS translocation from the gut to the circulation benefits from a high-fat diet, because this may increase the permeability of the gut epithelium and elevate the chylomicron levels (Cani et al. 2007; Musso et al. 2011). Relatively small intervention studies in humans in a controlled environment have reported an influence of the diet on circulating LPS levels: a high-fat and energy-rich diet may induce endotoxemia and thereby low-grade inflammation (Erridge et al. 2007; Ghanim et al. 2009). Also, it has been hypothesized that genetic factors and other dietary components, such as pH or salt content, might play a role in LPS translocation from the gut (Neves et al. 2013). Lifestyle, dietary habits, and the use of antimicrobial agents may affect the variety of bacterial species and the microbial load, modulating the composition of commensal microbiota and resulting in metabolic endotoxemia, which may also exist in apparently healthy subjects (Cani et al. 2007; Neves et al. 2013). However, the lack of clinical oral health examination in studies investigating endotoxemia complicates the interpretation of results.

Study IV of this thesis demonstrated that endotoxemia was strongly associated with prevalent obesity, MetS, diabetes, and CHD independently of established cardiometabolic risk factors, factors affecting serum LPS activity, and most importantly, energy or macronutrient intake. In addition, high serum endotoxin activity was associated with an elevated risk of incident CHD events in a ten-year follow-up. A direct association between endotoxemia and energy intake, and an indirect association with carbohydrate intake were detected, but these associations were only observed in healthy, lean subjects. Interestingly, obesity modifies the long-term associations between systemic inflammation and periodontitis. It has been shown that periodontitis affects systemic inflammation in a significant dose-dependent manner in lean subjects, but not in obese subjects (Gocke et al. 2014). This may suggest that endotoxemia and low-grade inflammation resulting from obesity and other cardiometabolic disorders is a persistent state, wherein periodontitis and a high-fat diet play only a minor role. In contrast to some previous studies (Erridge et al. 2007; Ghanim et al. 2009), no association between endotoxemia and fat intake was observed in this thesis. These results are in agreement with a very recent feeding trial,

which demonstrated no significant impact of fat intake on serum LPS activity in healthy subjects or patients with type 1 diabetes (Lassenius et al. 2014). However, our study design differed from feeding trials, since the fasting state serum LPS activity was analyzed instead of postprandial endotoxemia. In study IV, the nutrition information was based on 24-h dietary recall, which may be skewed due to random diet variations preceding the blood sampling. Long-term follow-up data on nutrition are needed to further analyze the systemic effects of nutrition on endotoxemia.

It has been shown that periodontitis is associated with body weight (Saxlin et al. 2011), and a dose-response relationship between BMI and the incidence of periodontitis has been reported (Morita et al. 2011). In mice, periodontitis itself could be potentiated by obesity induced by a high-fat diet via systemic inflammation (Blasco-Baque et al. 2012). The lack of clinical oral health examination is an important limitation of study IV. A detailed periodontal examination would have further enlightened the relationship between periodontitis and cardiometabolic disorders. However, only information on the missing teeth at the baseline was collected from the present population-based cohort. Intriguingly, the number of missing teeth as a marker for the history of periodontitis was shown to be associated with an increased risk of incident CVD events, incident diabetes, and all-cause mortality (Liljestrand et al. 2014).

5.7. Challenges in the determination of LPS activity

Serum endotoxin activity levels are widely measured by the use of the commercially available *Limulus* amebocyte lysate assay, which is the method of choice out of three basic LAL test methodologies available for the detection of endotoxin (Hurley 1995). LAL is an aqueous extract of blood cells called amoebocytes from the horseshoe crab (*Limulus Polyphemus)*, which is extremely sensitive to the presence of endotoxin. However, there are some challenges in measuring LPS activities with the LAL assay. One needs to appreciate that the measurement itself demands exhaustive care, since the contamination of environmental endotoxin in any solution or vessel is a common cause of experimental error.

It has been shown *in vitro* that LPS is able to activate leukocytes in the circulation in the pg/ml range (Nakagawa et al. 2002). The mean (SD) LPS activities measured from the thesis study populations were 44.0 (17.0) EU/ml in study II and 63.6 (37.2) EU/ml in study IV. These activities were in the same range as activities determined, for example, from Finnish healthy blood donors (35.9 EU/ml) (Pradhan-Palikhe et al. 2010) and middle-aged subjects (122.8 EU/ml) ([c] Pussinen et al. 2007). Furthermore, Goto et al. reported an LPS concentration of 6.7 pg/ml in healthy elderly subjects (Goto et al. 1994), and Pearson et al. determined an LPS concentration of 850 pg/ml in patients with Gram-negative sepsis

(Pearson et al. 1985). In addition to the variety of units reported and selection of assay kits and standardization available, there are even differences among the assay lots. Therefore, it is complicated to compare the results of LPS measurements between studies.

There is a lack of techniques to determine the serum LPS activity derived from specific periodotopathogens, since the LAL assay is not species-specific for any bacteria. LPS originates from Gram-negative bacteria from various sources via several routes, including commensal microbiota, the diet, and different bacterial infections, and the LAL assay measures the mixture of LPS in the sample. Therefore, the true origin of serum endotoxin activity in the studies remains unidentified. It has been shown that periodontitis patients suffer from endotoxemia (Silver et al. 1977; Geerts et al. 2002; [c] Pussinen et al. 2004), but a specific technique to measure LPS concentrations derived from periodontopathogens needs still to be established.

6. CONCLUSIONS

The aim of the thesis research was to investigate whether LPS links periodontitis with cardiometabolic disorders. The following topics were examined: the genetics predisposing to periodontitis (I), the systemic effects of endotoxemia induced by periodontitis (II, III) and cardiometabolic disorders (IV), as well as the influence of periodontal treatment on plasma LPS activity and lipoprotein composition (II, III).

It was demonstrated that a haplotype comprising six SNPs of the *BAT1*, *NFKBIL1*, and *LTA* genes was associated with the risk of periodontitis. The risk haplotype showed an association with BOP, PPD ≥6 mm, and severe periodontitis, and this discovery was replicated in two different study populations with concordance. To study the systemic effects of the polymorphisms, the serum LTA concentrations were measured. High serum LTA concentrations were associated with the *LTA* SNPs of the risk haplotype in homozygous patients. In addition, LTA was expressed in the inflamed periodontal tissue. The human MHC region has been shown to be significant in both innate and adaptive immunity, wherein LTA, stimulated by LPS, may further potentiate the systemic effects of periodontitis. Therefore, genetic variation in the MHC class III region may be important in the pathogenesis of periodontitis.

In periodontitis patients, LPS was associated with the proatherogenic VLDL-IDL fraction. Although local healing of the periodontium was successful after periodontal treatment, the systemic inflammation status failed to improve in all periodontitis patients, reflecting the persistence of the disease. There were no significant changes in plasma LPS activity or its distribution among lipoprotein classes after the periodontal treatment. However, the VLDL of the patients with severe periodontitis induced higher expression of proinflammatory cytokines in macrophages when compared with VLDL derived from the patients with moderate periodontitis. In addition, VLDL isolated from patients with severe periodontitis with suppuration contained more LPS and induced higher cholesterol uptake in macrophages *in vitro*. Endotoxemia and low-grade inflammation originating from periodontitis may promote the proatherogenic properties of VLDL particles to induce macrophage activation and foam cell formation. Thus, the thesis results suggest that LPS is involved in periodontitis-induced atherogenesis.

The study with a large, population-based cohort indicated that endotoxemia is strongly associated with prevalent cardiometabolic disorders, i.e. obesity, MetS, diabetes, and CHD. In addition, high serum LPS activity is associated with an increased risk of future CHD events. The results support the role of bacterial infections and the immune response in the etiology of cardiometabolic disorders. Even though energy intake was correlated with LPS activity in lean, healthy subjects, these associations were generally independent of

energy or macronutrient intake, emphasizing other LPS sources in addition to the gut. Since endotoxemia triggers low-grade inflammation, the findings of the present thesis support the role of LPS acting as a link between periodontitis and cardiometabolic disorders (**Figure 12**). In the future, better understanding of the molecular mechanisms related to LPS-mediated pathways may provide novel information for estimating the individual risk of cardiometabolic disorders.

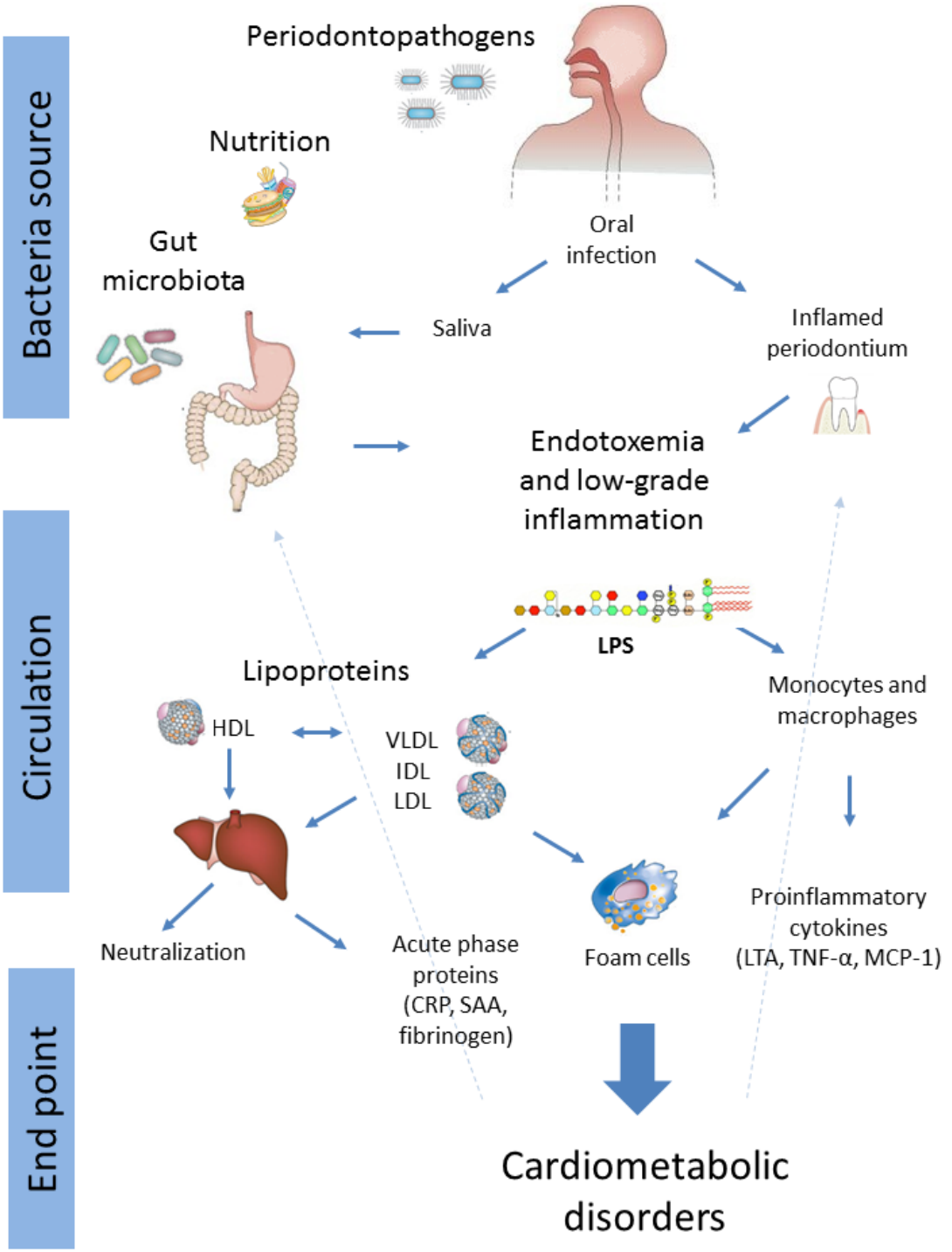

Figure 12. **Schematic representation of lipopolysaccharide acting as a link between periodontitis and cardiometabolic disorders.** LPS, lipopolysaccharide; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; SAA, serum amyloid A; LTA, lymphotoxin-α; TNF-α, tumor necrosis factor α; MCP-1, monocyte chemoattractant protein-1.

ACKNOWLEDGEMENTS

This thesis research was carried out during the years 2007–2014 at the Institute of Dentistry, Biomedicum Helsinki, University of Helsinki, Helsinki in collaboration with the National Institute for Health and Welfare, Helsinki. I wish to express my gratitude to the large number of people who have contributed to these studies, and helped and guided me during these years.

Professor Jarkko Hietanen, the former Dean of the Institute of Dentistry, Docent Hanna Thorén, the present Dean of the Institute of Dentistry, and Kirsti Kari, MSc, the head of the scientific laboratory of the Institute of Dentistry are acknowledged for providing excellent research facilities. The funding sources, the Aarne Koskelo Foundation, the Ida Montin Foundation, the Finnish Dental Society Apollonia, the Academy of Finland, the Sigrid Juselius Foundation, and the Doctoral Programme in Biomedicine, are acknowledged for making this work possible.

I wish to express my profound gratitude to my supervisor, Docent Pirkko Pussinen. Pirkko, your curiosity, open-mindedness, and insightful character have been a great inspiration to me. I could not have hoped for a better environment to grow as a researcher. I always knew I could come to you with any question, either related to research or not. You have succeeded in creating a group of people who are both passionate about science and keen to have fun. I'm proud to be a part of our group! Thank you for being understanding with the demands of combining research first with studies in dental school and later with clinical work.

I most sincerely thank my co-supervisor, Docent Matti Jauhiainen, for his exceptional passion and enthusiasm for science. Matti, your immeasurable ideas and endless energy have been motivating and inspiring me through these years. I greatly appreciate your knowledge in the area of lipoprotein metabolism.

I wish to warmly thank Professor Mikael Skurnik and Docent Susanna Paju for being in my thesis committee and the feedback and help during my PhD studies. I'm truly grateful to Professor Stina Syrjänen and Professor Olavi Ukkola for their deeply appreciated time, expertise, and constructive comments in revising this thesis. I would like to express my gratitude and honor to Professor Philippe Bouchard for accepting the invitation of being my official opponent, and Professor Timo Sorsa for accepting to act as a *custos* of my dissertation and guiding me into the academic world.

I'm also very grateful to all the collaborators I have had during my thesis project. I warmly thank my co-authors, Anita Tuomainen, Kåre Buhlin, Björn Klinge, Anders Gustafsson,

Ritva Keva, Kati Hyvärinen, Petri Kovanen, Päivi Mäntylä, Juha Sinisalo, Marja-Liisa Lokki, Efthymia Vlachopoulou, Anna Liisa Suominen, Marja Marchesani, Marcela Hernández, Markku Nieminen, Markus Perola, Markku Lehto, Satu Männistö, Veikko Salomaa, and Katja Hätönen. It has been a privilege to be able to work with you all. Without you this thesis would not exist.

I also want to express my warmest gratitude to all my co-workers at the Institute of Dentistry, both past and present. I especially want to thank my dear and talented colleagues Kati Hyvärinen, PhD, and Aino Salminen, DDS, MSc (Tech), for their friendship during these years. It has always been pleasant to enter our office! Thank you for all the help along the way, whatever the matter at hand, and all the fun adventures in and outside the lab, and also abroad. In addition, I wish to thank Anne Kivimäki, Mikko Nieminen, Abdirisak Ahmed, Laura Lahdentausta, John Liljestrand, Heidi Heikkola, Immi Kormi, and Maarit Takatalo-Laine for the coffee breaks, diverse discussions, and great company. Ritva Keva, Airi Sinkko, and Saija Perovuo are acknowledged for their excellent laboratory assistance.

I wish to extend my thanks to the Biotechnology Girls, Minnamari, Maria, Jenni, and Heini, for all the unforgettable moments during the last ten (!) years. We have shared thoughts and feelings about all sorts of aspects of science, PhD studies, and life in general. I also want to thank my friends outside the research circle, I'm truly grateful to have such wonderful people in my life!

My deepest thanks go to my family – my support team. You have always had faith in me and everything I do. Finally, Jukka, thank you for being there for me. Your love has made all the difference.

Elisa Kallio

Helsinki, October 2014

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