JAANA RENKO

Bacterial DNA Signatures in Arterial Inflammation

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ACADEMIC DISSERTATION
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To my family

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LIST OF ORIGINAL COMMUNICATIONS

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

I *Renko J*, Kalela A, Karhunen PI, Helin H, Sillanaukee P, Nikkari S, Nikkari ST (2003): Do temporal arteritis lesions contain bacterial DNA? Eur J Clin Invest 33: 657- 61.

II *Lehtiniemi J*, Karhunen PJ, Goebeler S, Nikkari S, Nikkari ST (2005): Identification of different bacterial DNAs in human coronary arteries. Eur J Clin Invest 35: 13- 6.

III *Renko J*, Lepp PW, Oksala N, Nikkari S, Nikkari ST (2008): Bacterial signatures in atherosclerotic lesions represent human commensals and pathogens. Atherosclerosis (in press).

IV *Renko J*, Kalela A, Jaakkola O, Laine S, Höyhtyä M, Alho H, Nikkari ST (2004): Serum matrix metalloproteinase-9 is elevated in men with a history of myocardial infarction. Scand J Clin Lab Invest 64: 255- 61.

In addition, some unpublished data are presented. The original articles are reproduced with the kind permission of Blackwell Publishing (I,II), Elsevier (III) and Taylor & Francis Group (IV).

ABBREVIATIONS

AAA	abdominal aortic aneurysm
ASO	atherosclerosis obliterans
ATCC	American Type Culture Collection
BHT	butylated hydroxytoluene
BLAST	basic local alignment search tool
BMI	body mass index
bp	base pair
Br-PCR	broad-range bacterial polymerase chain reaction
CAD	coronary artery disease
CHD	coronary heart disease
CRP	C-reactive protein
C3	C3-complement
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
HDL	high density lipoprotein
HSA	human serum albumin
IHD	ischemic heart disease
LAD	left anterior descending coronary artery
LDL	low density lipoprotein
LSU	large subunit
MI	myocardial infarction
MMP	matrix metalloproteinase
natLDL	native low density lipoprotein
OD	optical density
oxLDL	oxidized LDL cholesterol
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rDNA	gene encoding ribosomal RNA

rRNA	ribosomal ribonucleic acid
SD	standard deviation
SMC	smooth muscle cell
SSU	small subunit
TA	temporal arteritis
TIMP(s)	tissue inhibitor(s) of matrix metalloproteinases
UV	ultra violet
VLDL	very low density lipoprotein

ABSTRACT

Atherosclerosis is a chronic disease of multifactorial origin that is characterized by retention of cholesterol in the arterial wall and subsequent narrowing of the arterial lumen. There is a chronic inflammatory process in the atherosclerotic plaque, which has a major role in plaque growth and rupture. Plaques that are prone to rupture have often numerous inflammatory cells and a thin fibrous cap. Inflammation plays an important role in progression of atherosclerosis and it is clear that bacteria take part in inflammatory processes. The most identified bacteria involved in atherosclerotic disease are *Chlamydia pneumoniae* and bacteria involved in dental infections.

The aims of this study were to investigate whether bacterial DNA is present in different human artery specimens by using broad-range 16S rDNA PCR methodology. This method is based on the use of primers that recognize conserved sequences of bacterial chromosomal genes encoding ribosomal RNA. Between these concerved regions, sequences also contain variable regions providing a reliable basis for the analysis of bacterial phylogenetic relationships. Human coronary artery-, abdominal artery-, and temporal artery samples were studied. We also developed a novel subtraction method to rule out potential methodological contaminants. In addition, serum matrix metalloproteinase-9 (MMP-9) and other established inflammatory markers were examined in men with coronary heart disease, who had suffered myocardial infarction.

The presence of bacterial DNA in atherosclerotic lesions suggests a multi-infection role of microbes in the atherosclerotic process. Serum MMP-9 concentration was elevated in men with a history of previous myocardial infaction compared to controls. The results underly the importance of inflammation –and possibly also infection– in the progression of atherosclerosis.

Keywords: atherosclerosis, inflammation, broad-range PCR, bacterial DNA, MMP-9

TIIVISTELMÄ

Valtimonkovettumatauti eli ateroskleroosi on monien tekijöiden aiheuttama krooninen tauti, jolle on ominaista kolesterolin kertyminen valtimon seinämään ja valtimon ontelon kaventuminen. Ateroomaplakissa vallitsee krooninen tulehdus, jolla on merkittävä rooli aterooman kasvamismekanismeissa ja repeämisessä. Repeämälle alttiissa plakeissa on usein paljon tulehdussoluja ja ohut sidekudoskatto. Inflammaatiolla on tärkeä rooli ateroskleroosin kehityksessä ja on ilmeistä, että bakteerit osallistuvat inflammaation syntyyn. Ateroskleroosiin liittyvistä bakteereista tunnetuimpia ovat *Chlamydia pneumoniae* ja hammasinfektioita aiheuttavat bakteerit.

Tutkimuksessa selvitettiin broad-range 16S rDNA polymeraasiketjureaktion (PCR) avulla, löytyykö bakteerien DNA:ta ihmisperäisistä valtimonäytteistä. Menetelmä perustuu sellaisten alukkeiden käyttöön, jotka tunnistavat bakteerien konservoituneita sekvenssejä ribosomaalista RNA:ta koodaavasta kromosomaalisesta geenialueesta. Näiden konservoituneiden alueiden välissä on variaabeleita alueita, joiden perusteella voidaan määrittää bakteerien fylogeneettisiä sukulaisuussuhteita. Sepelvaltimo-, vatsa-aortta- ja ohimovaltimonäytteet olivat tutkimuksen kohteena. Kehitimme myös subtraktioanalyysin, jolla metologisia kontaminantteja voidaan välttää. Lisäksi tutkimuksessa mitattiin seerumin matriksin metalloproteinaasi-9 (MMP-9) -pitoisuutta ja muita tulehdustekijöitä sellaisilta sepelvaltimotautipotilailta, jotka olivat sairastaneet sydäninfarktin.

Ateroskleoottisissa plakeissa oli bakteerijälkiä taudinaiheuttajista, joka tukee ateroskleroosin multi-infektioteoriaa. Seerumin MMP-9 pitoisuus oli koholla sydäninfarktin sairastaneilla potilailla verrattuna kontrolleihin. Tulokset osoittavat, että valtimon seinämän tulehdus – ja mahdollisesti myös bakteeri-infektiot – ovat yhteydessä ateroskleroosin kehitykseen.

Avainsanat: ateroskleroosi, inflammaatio, broad-range PCR, bakteeri DNA, MMP-9

INTRODUCTION

Atherosclerosis is a disease of large and medium-sized arteries which leads to intimal thickening and loss of arterial elasticity. The disease often begins in childhood (Stary 1989, PDAY research group 1993), but clinical symptoms may not appear until middle age or later when the arterial lesions precipitate organ injury. The clinical complications of atherosclerosis, including myocardial infarction and stroke, are the major cause of morbidity and mortality among men in the developed world (Ross 1999b).

Inflammation has been shown to play an important role in the clinical complications of atherosclerosis. The activated macrophages abundant in atheroma can produce proteolytic enzymes, such as matrix-degrading metalloproteinases (MMPs), capable of degrading the collagen that lends strength to the plaque's protective fibrous cap, rendering the cap thin, weak, and susceptible to rupture (Dollery *et al.* 1995, Galis & Khatri 2002). Plaque disruption takes place often at the shoulder region between the plaque and the adjacent vessel wall where the fibrous cap is the thinnest (Richardson *et al.* 1989). The inflammation in atherosclerosis has been associated with infection by various bacterial and viral pathogens (Epstein *et al.* 1999). Pathogens that have been implicated include *Chlamydia pneumoniae*, cytomegalovirus, herpes simplex virus, *Helicobacter pylori*, and periodontal infections (for reviews, see Epstein *et al.* 1999, Fong 2000, Leinonen & Saikku 2002). Other classical risk factors of atherosclerosis are high serum cholesterol, hypertension, diabetes mellitus, and smoking (Vartiainen *et al.* 1994, Burke *et al.* 1997, Wilson *et al.* 1998).

Ample evidence suggests that infectious agents may play a role in the pathogenesis of atherosclerosis and in the clinical manifestations of vascular disease. However, most of the studies have concentrated on the potential role of only one specific microbe, such as *C. pneumoniae* (Campbell *et al.* 1995, Ramirez 1996) or *H. pylori* (Ameriso *et al.* 2001, Kaplan *et al.* 2006). Less frequently, a broader multi-microbial presence in the etiology of the atherosclerotic plaque has been suggested (Watt *et al.* 2003, Ott *et al.* 2006). This thesis aimed at studying which bacterial signatures are present in human atherosclerotic arterial specimens as well as

demonstrating the presence of MMP-9 and other inflammatory markers from sera of subjects with complications of atherosclerosis.

REVIEW OF THE LITERATURE

1. The structure of blood vessels

The artery wall has three layers, the intima, the media, and the adventitia (Stary *et al.* 1992). A single layer of endothelial cells covers the luminal side of the intima. The intima consists of a narrow subendothelial space containing smooth muscle cells (SMC), but also macrophages, T-lymphocytes, and mast cells may be present. The intima is separated from the media by the internal elastic lamina, a fenestrated sheet of collagen. The media is the thickest layer, responsible for the structural integrity of the arterial wall. The outermost layer is the adventitia, which consists mainly of fibroblasts and connective tissue together with nerves and small blood and lymph vessels, termed vasa vasorum. The media and the adventitia are separated by the external elastic lamina (Geer & Haust 1972, Stary *et al.* 1992).

2. Classification of atherosclerotic lesions

Atherosclerosis begins early in life with accumulation of low density lipoprotein (LDL) cholesterol and monocytes in the intima of large arteries (McGill & McMahan 1998). LDL is trapped in the intimal extracellular matrix, or it passes through the internal elastic lamina and moves further into the media (Williams & Tabas 1995, Williams & Tabas 1998, Kadar & Glasz 2001, Skålen *et al.* 2002). In the intima, LDL may be taken up by monocyte-derived macrophages (Witztum & Steinberg 1991) leading to foam cell formation. The earliest lesion, the fatty streak, is primarily caused by the accumulation of lipid droplets in macrophages (Stary *et al.* 1994). A part of the fatty streaks are disposed to progression and may proceed to intermediate lesions and further to advanced lesions such as the atheroma, fibroatheroma, and complicated fibroatheroma (Stary *et al.* 1994).

Since the 1990s, a specific classification of atherosclerotic lesions has been established by the Committee on Vascular Lesions of the Counsil of Atherosclerosis, American Heart Association (Stary *et al.* 1992, Stary *et al.* 1994, Stary *et al.* 1995).

This classification is based on histology and it divides the lesions into early and advanced types.

The early lesions are classified as types I, II, and III. Type I lesions contain macrophages with intracellular lipid droplets (foam cells) without other significant histological changes (Stary *et al.* 1994). Type II lesions are those lesions that are also known as fatty streaks. Fatty streaks may be seen on the inner surfaces of arteries as relatively flat, yellow colored streaks or patches (Stary et al. 1994). They consist mainly of macrophage foam cells, but intimal SMCs may contain lipid droplets as well. Fatty streaks do not obstruct arterial blood flow. Type III lesions form the bridge between minimal and advanced lesions as they contain extracellular lipid droplets that accumulate into small pools lying below the macrophage layer (Stary *et al*. 1994).

Advanced atherosclerotic lesions are referred to as type IV – VI. The type IV lesion is the atheroma. It may not cause any symptoms, and these lesions are often not visible by angiography. These lesions contain an accumulation of extracellular lipid in the intima, known as the lipid core. The major cell types on top of the lipid core are macrophages and SMCs with and without intracellular lipid (Stary *et al.* 1995). There are also mast cells and lymphocytes in this region (Kovanen *et al.* 1995, Kaartinen *et al.* 1998). These plaques have a thin cap of connective tissue above the lipid core, which makes the plaque prone to rupture. Type V lesions have a prominent fibrous cap of connective tissue between the lipid core and endothelium. This type of lesion is referred to as a fibroatheroma (or type Va lesion). A type V lesion, in which the lipid core or other parts of the lesion are calcified, is called a calcified lesion (or type Vb lesion). A type V lesion in which the lipid core is absent and lipid in general is minimal may be referred as type Vc. These lesions cause various narrowing of arteries. The type V lesions are prone to progress to type VI lesions by rupturing and subsequent formation of mural thrombi, causing further narrowing of the arterial lumen. Type VI complicated lesions have a disrupted structure including hematoma (type VIa), hemorrage (type VI b), or thrombosis (type VIc). These lesions cause acute clinical manifestations of atherosclerosis (Stary *et al.* 1995).

The present study examined lesions ranging from fibroproliferative thickening observed in temporal arteritis (TA) to complicated atherosclerotic lesions seen in coronary artery disease and abdominal atherosclerosis. TA is a disease characterized by acute inflammation in medium to large arteries in elderly people, especially females (Bengtsson & Malmvall 1981, Nordborg *et al.* 2000, Salvarani *et al.* 2002a). An environmental infective trigger for TA has long been supposed on the basis of the clinical presentation as flu-like symptoms are frequently seen at the onset of disease and are followed by a marked acute-phase response (Larson *et al.* 1984).

Abdominal aortic aneurysm (AAA) is a focal, balloon-like dilation of the terminal aortic segment that affects the infrarenal portion of the abdominal aorta. AAA is most likely related to systemic atherosclerosis since the diseases share risk factors (Johnson *et al.* 1985, Reed *et al.* 1992, Van der Vliet & Boll 1997). Arteriosclerosis obliterans (ASO) is another characteristic disorder of atherosclerosis, in which arterial obstruction occurs in arteries supplying blood to lower extremities (Second European Consensus Document on chronic critical leg ischemia 1991).

3. Infection, inflammation and atherosclerosis

3.1. The role of inflammation in atherosclerosis

Numerous inflammatory mediators play a role in early atherosclerotic lesion formation. They take a part in the early events including attracting monocyte-leukocytes from circulating blood by vascular endothelial cells leading to their migration into the intima, transitioning monocytes into macrophages and eventually into lipid-laden foam cells (Ross 1999a,b, Libby *et al.* 2002, Järvisalo *et al.* 2006). Later in the process, inflammatory mediators can weaken the protective fibrous cap of the atheroma possibly leading to thrombosis and the occurrence of acute cardiovascular events such as unstable angina pectoris and myocardial infarction (MI) (Ross 1999a,b, Libby *et al.* 2002).

3.1.1 C-reactive protein

C-reactive protein (CRP), an acute phase protein which is named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*, is considered to be the most valuable inflammatory marker to predict cardiovascular events, including myocardial infarction, stroke, and vascular mortality (Haverkate *et al.* 1997, Ridker *et al.* 1998a, Ferreiros *et al.* 1999, Lindahl *et al.* 2000, Rost *et al.* 2001, Ridker *et al.* 2002). It is synthesized mainly by hepatocytes under the control of several cytokines, with interleukin-6 being a primary stimulus (Pepys & Hirschfield 2003). Although the principal source of CRP is the liver, recent data have shown that vascular tissue itself can also produce CRP (Yasojima *et al.* 2001, Ishikawa *et al.* 2004, Wilson *et al.* 2007). The major producers of CRP in atherosclerotic lesions are smooth muscle cells and macrophages (Yasojima *et al.* 2001). In addition to predicting recurrent cardiovascular events in patients with atherosclerotic disease, elevated levels of CRP are one of the strongest predictors of progressive vascular disease (Van der Meer *et al.* 2002) and future cardiovascular events in apparently healthy men and women (Ridker *et al.* 1997, Ridker *et al.* 1998b, Koenig *et al.* 1999).

3.1.2 Antibodies of oxidized LDL

Oxidized LDL cholesterol (OxLDL) has a significant role in the progression of atherosclerosis (Steinbrecher & Pritchard 1989, Parthasarathy *et al.* 1990, Parthasarathy & Rankin 1992). OxLDL accumulates in macrophages, accelerates the development of foam cells and stimulates the monocyte adhesion to endothelium (Witztum 1991, Witztum & Steinberg 1991, Jialal & Devaraj 1996). In addition, oxLDL stimulates the synthesis of MMP-9 in macrophages by increasing the expression of mRNA and protein synthesis, influencing possibly the rupture of atherosclerotic plaques (Xu *et al.*1999). The small, dense LDL particles are more susceptible to oxidation, which mainly takes place in the arterial wall (Krauss 1995, Galeano *et al.* 1998)

OxLDL is also immunogenic and it induces the formation of autoantibodies (Palinski *et al.* 1989, Palinski *et al.* 1994, Ylä-Herttuala *et al.* 1994, Palinski *et al.* 1996). The antibody titers of oxidized LDL have been shown to correlate with the severity of atherosclerosis and it has been thought to predict myocardial infarction (MI) (Salonen *et al.* 1992, Puurunen *et al.* 1994, Wu *et al.* 1997). However, contradictory results have also been reported. A study by van de Vijver and her colleagues (van de Vijver *et al.* 1996) did not discover any association between oxLDL autoantibodies and the extent of coronary stenosis. In addition, lower oxLDL autoantibody levels in elderly patients with ischemic stroke (Cherubini *et al.* 1997) and in acute MI compared to controls have been reported (Schumacher *et al.* 1995).

3.1.3 Matrix metalloproteinase-9

Activated cells in inflamed atherosclerotic plaques, such as macrophages, smooth muscle cells, and endothelial cells, secrete matrix-degrading proteases including matrix metalloproteinases (MMPs). Elevated levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-12 have been observed in atherosclerotic plaques (Henney *et al.* 1991, Galis *et al.* 1994a, Brown *et al.* 1995, Nikkari *et al.* 1995, Halpert *et al.* 1996, Li *et al.* 1996). Vascular cells also produce tissue inhibitors of matrix metalloproteinases (TIMPs) that inhibit MMP activity (Galis *et al.* 1995).

MMP-9, also known as gelatinase B or 92 kDa gelatinase, is associated with arterial inflammation. It has been indicated to be highly expressed in the disruption-prone regions of atherosclerosic plaques (Galis *et al.* 1994a, Brown *et al.* 1995, Zaltsman & Newby 1997) whereas non-atherosclerotic arteries have not been shown to contain detectable amounts of MMP-9 (Brown *et al.* 1995). Macrophages are the major source of MMP-9 although a variety of other cell types also express this protease (Galis *et al.* 1994b, Brown *et al.* 1995, Kaartinen *et al.* 1998, Nelimarkka *et al.* 1998). Increased serum levels of MMP-9 have been detected both in stable and unstable CAD (Kai *et al.* 1998, Inokubo *et al.* 2001, Noji *et al.* 2001).

3.2 The role of infection in atherosclerosis

Microorganisms may play a role in different stages of atherogenesis by numerous mechanisms of action. They may infect vascular endothelium directly, thus initiating the inflammatory response to induce atherogenesis (Libby *et al.* 1997, Nieto 1998, Albert 2000, Fong 2000, O'Connor *et al.* 2001, Fong 2002). In addition, although the induction or initial injury to the endothelial wall would be caused by another agent or factor such as hypercholesterolemia or hypertension, microorganisms enhance atherogenesis through number of mechanisms. These include recruitment and stimulation of proinflammatory cytokines and tissue growth factors in the arterial wall (Coombes & Mahony 1999, Summersgill *et al.* 2000), as well as enhancement of LDL accumulation through stimulation of macrophage scavenger- or LDL-receptors (Zhou *et al.* 1996, Kalayoglu & Byrne 1998, Epstein *et al.* 1999, Fong 2002). Microorganisms could also indirectly influence the development of atherosclerosis by systemic changes through initiation of immunological responses to microbial antigens, which crossreact with normal human antigens (Libby *et al.* 1997, Muhlestein 1998, Nieto 1998, Albert 2000). The most frequently studied bacteria for their possible role in the process of atherosclerosis are *Chlamydia pneumoniae*, *Helicobacter pylori* and bacteria involved in dental infections (for reviews, see Fong 2000, Leinonen & Saikku 2002, Muhlestein & Anderson 2003).

3.2.1 *Chlamydia pneumoniae*

Chlamydia pneumoniae, a gram-negative obligate intracellular bacterium, is a widespread respiratory pathogen. After the first report by Saikku and colleagues in 1988 (Saikku *et al.* 1988), several seroepidemiological and other studies have demonstrated both positive and negative associations between chronic *C. pneumoniae* infection and atherosclerosis. Strong associations have been verified by: (a) seroepidemiological surveys showing that patients with cardiovascular disease have higher titres of anti-*C. pneumoniae* antibodies compared with control patients (Saikku *et al.* 1992, Romano Carratelli *et al.* 2006); (b) detecting the microbe within atheroma but not in adjacent normal tissue by electron microscopy, immunocytochemistry, and polymerase chain reaction (PCR) (Kuo *et al.* 1993,

Grayston *et al.* 1995, Ramirez 1996, Jackson *et al.* 1997) and by culturing the microbe from atherosclerotic tissue (Ramirez 1996, Maass *et al.* 1998); and (c) showing that *C. pneumoniae* can either initiate development of atherosclerosis or generate exacerbation of lesions in rabbit and mouse animal models, respectively (Fong *et al.* 1997, Laitinen *et al.* 1997, May *et al.* 2003). Numerous studies have also failed to: (i) confirm the association between antibodies to *C. pneumoniae* and cardiovascular disease (Altman *et al.* 1999, Nobel *et al.* 1999, Chandra *et al.* 2001, Kähler *et al.* 2001) (ii) detect *C. pneumoniae* in atherosclerotic lesions by PCR (Weiss *et al.* 1996, Ong *et al.* 2001, Apfalter *et al.* 2004, Maraha *et al.* 2004); (iii) detect increased lesion development after *C. pneumoniae* infection in ApoE-deficient mice (Aalto-Setälä *et al.* 2001, Caligiuri *et al.* 2001). In addition, some meta-analyses of prospective studies have not provided strong evidence of an association between atherosclerosis and *C. pneumoniae* (Danesh 1999, Danesh *et al.* 2000).

3.2.2 Helicobacter pylori

Helicobacter pylori is a gram-negative bacterium whose only natural niche is in the human stomach, where it resides in the mucus layer and on the gastric epithelium (Dunn *et al.*1997). The association between *H. pylori* and atherosclerosis was first suggested by Mendall and his colleagues in 1994 (Mendall *et al.* 1994). Since that, a number of seroepidemiological studies have suggested that patients with ischemic heart disease (IHD) have higher titers of anti-*H. pylori* antibodies compared to controls (Pasceri *et al.* 1998, Danesh *et al.* 1999a, Pellicano *et al.* 1999, Alkout *et al.* 2000) although previous published studies are inconsistent (Folsom *et al.* 1998, Strachan *et al.* 1998, Zhu *et al.* 2002). In addition, molecular studies investigating the presence of *H. pylori* in atherosclerotic plaques by PCR are contradictory. Several studies have not been able to detect *H. pylori* from atherosclerotic samples (Blasi *et al.* 1996, Malnick *et al.* 1999, Danesh *et al.* 1999b, Dore *et al.* 2003, Weiss *et al*. 2006) whereas others have reported its presence (Farsak *et al.* 2000, Ameriso *et al.* 2001, Kaplan *et al.* 2006).

3.2.3 Dental infections

Gram-negative bacteria, including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Prevotella intermedia*, are related with gingivitis and periodontitis (Williams 1990) whereas gram-positive bacteria, particularly *Streptococcus mutans*, are related with etiology of caries (Loesche 1986). Mattila and his colleaques (Mattila *et al.* 1995) were the first to show that dental infections evaluated by the pantomography index were significantly more common in patients with advanced coronary atherosclerosis. After that, DNA of periodontitis-related bacteria has been identified in different atheromatous samples in several recent studies (Haraszthy *et al.* 2000, Okuda *et al.* 2001, Stelzel *et al.* 2002, Taylor-Robinson *et al.* 2002, Kurihara *et al.* 2004, Fiehn *et al.* 2005). *S. mutans* has been detected in atheromatous plaque specimens as well (Kozarov *et al.* 2006, Nakano *et al.* 2006). Furthermore, a recently published study by Kozarov *et al.* (2005) demonstrated signs of viable *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human atherosclerotic plaque material by using cell culture invasion assays and immunofluorescent microscopy. The association between periodontal microbes and atherosclerosis is speculated by the fact that the oral cavity is well vascularized, and the occurrence of infection in this site provides an instant and direct route to the bloodstream. Professional dental manipulations and daily oral care practices, such as tooth brushing and flossing, lead to seeding of the oral flora to the bloodstream (Silver *et al.* 1977, Heimdahl *et al.* 1990, Roberts *et al.* 1997, Seymour *et al.* 2000, Bhanji *et al.* 2002, Rajasuo *et al.* 2004, Forner *et al.* 2006). However, even though many epidemiological studies have indicated significant relations between periodontal diseases and atherosclerosis (Mattila *et al.* 1995, Beck *et al.* 1996, Joshipura *et al.* 1996, Morrison *et al.* 1999), there are some epidemiological studies that have not been able to show such relations (Hujoel *et al.* 2000, Howell *et al.* 2001).

4. Molecular identification of bacteria

4.1 rRNA molecules

Ribosomes, which are large complex ribonucleotide particles, are composed of several different ribosomal RNA (rRNA) molecules and over 50 proteins, organized into a large (LSU) and small (SSU) subunits. Homologous SSU and LSU major rRNA molecules can be found in all bacteria, where they play a role in protein synthesis (Noller 1984, Gutell *et al.* 1994, Lodish *et al.* 2000, Lewin 2004). Prokaryotic SSU has a sedimentation value of approximately 30S consisting one major RNA molecule and 21 proteins. Prokaryotic LSU has a sedimentation rate of about 50S consisting one major and one minor RNA molecule and 31 different protein molecules (Lodish *et al.* 2000, Berg *et al.* 2002, Lewin 2004). rRNA molecules are named according to their sedimentation rate. In prokaryotes, the rRNA molecule of SSU is named as 16S rRNA; the LSU containing two rRNA molecules are named 23S rRNA and 5S rRNA. 16S and 23S rRNA molecules are of large size: about 1540 and 2900 bases, respectively (Lodish *et al.* 2000, Lewin 2004).

4.2 Bacterial taxonomy

Bacterial taxonomy, or systematics, may be defined as a scientific study of the diversity of organisms and their relationships (Rademaker & Savelkoul 2004). Nomenclature, classification, and identification are its three parts. The purpose of classification is to define the characteristics of a taxon and its position in the hierarchical system. This information is used when a new isolate is defined before it can be identified as a member of a known taxon. Nomenclature governs the taxonomic names to the taxa e.g. according to the *International Code of Nomeclature of Bacteria* (Sneath 1992). In identification, members of different taxonomic unit are named on the basis of characteristics or properties of the organism that distinguishes them from others (Rademaker & Savelkoul 2004).

Traditionally, classification and identification of bacteria in the clinical laboratory have been based on isolation of bacterial strains as pure culture and analysis of the isolate´s phenotypic characters such as certain biochemical properties (Kloos & Schleifer 1975, Schleifer & Kloos 1975). No phylogeny-based classification scheme of bacteria existed until the rRNA sequence-based methods were introduced (Woese 1987).

4.3 Broad-range rRNA approach

The phylogenetic classification of microorganisms based on rRNA molecule sequences offers a distinct framework which can be used to develop molecular tools for detection and identification of microbes (Stahl & Amann 1991, Stahl & Kane 1992). The rRNA genes have well conserved domains which are suitable as sites for PCR primers that recognize large groups of organisms, and variable domains that provide signatures for more precise identification at phylogenetic levels below the level originally targeted by the primers (Gutell *et al.* 1985, Lane *et al.* 1985, Woese 1987, Maiwald 2004). An overview of both conserved and variable regions in the bacterial 16S rRNA gene is given in Figure 1. Thus, the power of broad-range rRNA PCR lies in the relative absence of selectivity, so that –at least in theory– any kind of bacterium present in a sample can be detected and identified without prior cultivation or any knowledge of its nature (Relman *et al.* 1990, Kroes *et al.* 1999, Maiwald 2004).

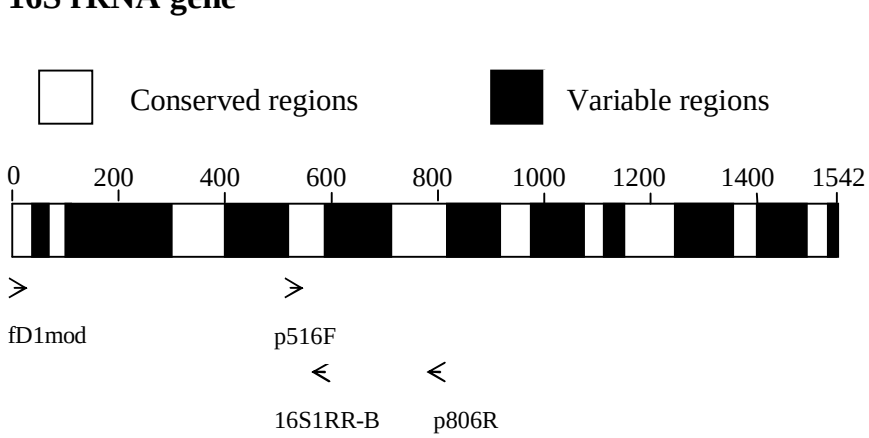

Figure 1. Schematic drawing of the bacterial 16S rRNA gene with conserved and variable regions. The location of the broad-range primers used in the present study (see also Table 2) is shown. (Modified from Maiwald 2004)

4.4 The rRNA approach on atherosclerotic diseases

The 16S rRNA-based PCR methodology has been used to study the suspected bacterial etiology of atherosclerosis (Haraszthy *et al*. 2000, Watt *et al*. 2003, Nakano *et al.* 2006, Marques da Silva *et al.* 2006, Ott *et al.* 2006). The results of these studies are summarized below.

Haraszthy *et al.* (2000) examined the presence of bacterial 16S rRNA as well as *C. pneumoniae* and human cytomegalovirus from 50 human specimens obtained during carotid endarterectomy by using PCR. They further assessed the presence of periodontal bacteria by using digoxigenin-labeled species-specific oligonucleotide probes for *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Porphyromonas gingivalis,* and *Prevotella intermedia*. Eighty percent of the 50 atheroma specimens were positive in one or more of the PCR assays. PCR of the bacterial 16S rDNA indicated the presence of bacteria in 72% of the atheroma specimens. Forty-four percent of 50 atheroma specimens were positive for at least one of the target periodontal bacteria. Watt *et al.* (2003) studied the presence of viral and bacterial DNA in carotid atherosclerotic lesions from 18 surgical specimens.

Specific primers for *C. pneumoniae*, cytomegalovirus, herpes simplex virus 1 and 2, and bacterial 16S rDNA were used in PCR assays. Only herpes simplex 1 DNA (3/18 specimens) and bacterial 16S rDNA (8/18 specimens) were detected from carotid atherosclerotic lesions. Detected bacterial DNA was shown to belong to the human flora (oral, genital, or faecal) or the environment. Nakano *et al.* (2006) analyzed the presence of streptococcal species in diseased heart valve (n=35) and atherosclerotic plaque (n=27) specimens, as well as in dental plaque samples (n=32) from the same subjects by using oral streptococcal species specific primer sets for the glucosyltransferase gene as well as broad range bacterial 16S rDNA PCR with direct sequencing. Streptococcal species were detected by both PCR methods. Especially *S. mutans* was significantly prevalent. Ott *et al.* (2006) studied 16S rDNA signatures in atherosclerotic tissue obtained through catheter-based atherectomy of 38 patients with CHD, control material from postmortem patients (n=15), and heart-beating organ donors (n=11) using clone libraries, denaturating gradient gel analysis, and fluorescence *in situ* hybridization. Bacterial DNA was found in all CHD patients by conserved PCR but not in control material or in any of the normal/unaffected coronary arteries. Presence of bacteria in atherosclerotic lesions was confirmed by fluorescence *in situ* hybridization. Over 50 different species were demonstrated in >1500 clones from a combined library and confirmed by denaturating gradient gel analysis. Several of the species detected in human coronaries in this study represented commensal human flora (skin, respiratory, and oral cavity). Marques da Silva *et al.* (2006) studied the presence of bacterial DNA in 10 specimens from arterial wall of aortic aneurysms. 27 different species were identified among 83 clones sequenced. These bacteria included oral as well as environmental bacteria. The results of these studies are summarized in Table 1.

Table 1. Broad-range 16S rRNA PCR-based studies for suspected bacterial etiology of atherosclerosis.

Reference	Specimens/ controls	Disease	Results
Haraszthy *et al.* 2000	50 / 0	Carotid stenosis	Specimens 72% positive; 44% positive for at least one periodontal bacteria
Watt *et al.* 2003	18 / 0	Carotid stenosis	Specimens 44% positive. Detected bacterial DNA from oral, genital, and faecal flora or environment
Nakano *et al.* 2006	27/ 0	Thoracic- or AAA	Specimens 89% positive for *Streptococcus* species
Ott *et al.* 2006	38 / 26	CHD	Specimens 100% positive, all controls negative. Detected bacterial DNA from skin, respiratory, and oral cavity
Marquez da Silva *et al.* 2006	10 / 0	Aortic aneurysm	Specimens 100% positive. Detected bacterial DNA from oral cavity and environment

AAA = abdominal aortic aneurysm; CHD= coronary heart disease.

AIMS OF THE STUDY

Inflammation is an important defense mechanism against infection. Chronic or recurrent infections may cause sustained inflammation that has been linked to increased risk of atherosclerosis. Several epidemiological, biological, experimental, and clinical studies have shown that infections may play a role in atherosclerosis and related diseases.

The aim of this study was to investigate whether bacterial DNA is present in arterial walls of different clinical specimen by using Br-PCR methodology. In addition, serum inflammatory markers in male subjects with a history of previous MI were compared to controls.

The spesific aims were:

1. to study whether bacterial DNA are present in temporal artery tissue of patients with temporal arteritis (TA).
2. to elucidate whether bacterial DNA is present in coronary specimens obtained from left anterior descending coronary arteries of subjects with sudden deaths of cardiovascular and other causes, as verified by autopsy.
3. to define bacterial DNA signatures in surgically removed sterile abdominal aorta samples of patients with aortic atherosclerosis.
4. to examine whether serum MMP-9 concentration may reflect inflammatory pathologic processes that are related to progression of atherosclerosis in subjects with a history of MI.

SUBJECTS, MATERIALS AND METHODS

1. Clinical specimens (I-III)

Temporal artery specimens (I) were collected from 27 patients with temporal arteritis (20 women, 7 men; mean age 75 years; range 61 to 99 years). Five uninvolved temporal arteries were also included (4 women, 1 man; mean age 64 years; range 54 to 80 years). A lung sample obtained from a 91 year old man, who died of sepsis and bacterial pneumonia following gastrointestinal surgery, acted as a positive control. Coronary specimens (II) were obtained from the proximal part of the main trunk of the left anterior descending coronary artery (LAD) of five subjects who died of sudden coronary causes (4 men, 1 woman; mean age 69 years; range 56 to 79 years) and five controls (4 men, 1 woman; mean age 34 years; range 26 to 48 years) within 3 days after death. At autopsy, the proximal part of the LAD was removed and immediately frozen at –70 °C. Abdominal aortic specimens (III) were obtained in abdominal aortic surgeries of 20 patients (18 men and 2 women; mean age 65 years; range 45 to 81 years) who had a medical history of atherosclerosis obliterans (n= 4) or who had suffered from abdominal aortic aneurysm (n= 16). None of the patients were known to have any clinical signs of infection in the days before the surgery. After surgical removal, the abdominal aortic tissue was placed in a sterile container, kept at +4 °C, and transported within the same day to the laboratory on ice. All patients considered for the study gave written informed consent before surgery. The study protocols were approved by the Ethical Committee of Tampere University Hospital.

2. Preparation of clinical tissue specimens (I-III)

2.1 DNA isolation from paraffin embedded temporal artery samples

Paraffin-embedded transverse sections of temporal arteries and lung sample were deparaffinized with xylene and ethanol. Tissues were digested with proteinase K (final concentration 0.1 mg/ ml) at 56 °C for 3 hours. Proteinase K was heat

inactivated (8 minutes at 95 °C) and the reaction mixture was centrifuged (10 minutes at maximum speed) and the supernatant was used for PCR analysis.

2.2 DNA isolation from coronary and abdominal aortic samples

Clinical specimens adjacent to the cryostat sections were digested with proteinase K (final concentration 0.1 mg/ ml) at 56 °C for 3-7 hours. After proteinase K treatment, the samples were heated 8 minutes at 95 °C to inactivate proteinase K, and then the reaction mixture was centrifuged (15 minutes at maximum speed) and the supernatant was used for PCR analysis.

3. Broad-range bacterial PCR (I-III)

3.1 PCR

Exact procedures for DNA amplifications are described in original communications (I-III). A location of used broad-range primers for 16S rDNA is shown in Figure 1. The total reaction volume was 50 µl, containing either 0.5 µl or 5µl of the supernatant from DNA extraction, 10 to 100 pmol/ µl of each primer (Table 2), and standard amounts of HotStarTaq Master Mix-reagent containing 2.5 units HotStarTaq DNA Polymerase, 15mM $MgCl_2$, and 200µM of each dNTP (Qiagen GmbH, Hilden, Germany). PCR reactions were performed in sterilized 500 µl thin wall tubes. Thermal cycling conditions are given in the original communications (I-III). PCR was performed using Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) (I) and TechGene (Techne, Burlington, USA) (II-III) thermal cyclers described in the original communications (I-III). Twenty eight to 50 thermal cycles were used.

Table 2. PCR and sequencing primers

Study	Primer	Sequence (5' to 3')	Location *	Use	Reference
III	fD1mod	agagtttgatc(tc)tgg(tc)t(tc)ag	8 27	16S PCR	Jalava *et al.* 1995
III	16S1RR-B	ctttacgccca(ag)t(ag)a(at)tccg	575-556	16S PCR	Bergmans *et al.* 1995
I-II	p516F	tgccagcagccgcggtaa	516-533	16S PCR	Relman *et al.* 1992
I-II	p806R	ggactaccagggtatctaat	806-787	16S PCR	Relman *et al.* 1992
II-III	M13(-20)F	gtaaaacgacggccag	391-406	Vector priming site	
II-III	M13R	caggaaacagctatgac	205-221	Vector priming site	
I-III	β-actin	tgactgactacctcatgaagatcctcaccg		#	Nikkari *et al.* 2001
I-III	β-actin	ccacgtcacacttcatgatggagttg		#	Nikkari *et al.* 2001

* The location numbers are positions in *Escherichia coli* 16S rRNA (Guttell *et al.* 1985) or pCR® 2.1-TOPO® vector (www.invitrogen.com).

Quality control of PCR inhibition or degradation of DNA.

3.2 Control of DNA isolation

As a quality control of the samples and the DNA extraction, the presence of human DNA was verified by amplifying a part of human β-actin gene (Table 2).

3.3 Gel electrophoresis

PCR products were analysed by using gel electrophoresis. Eight µl of the amplified product was separated by 1.5- 2.0 % LE analytical grade (Promega, Mannheim, Germany) agarose gel containing ethidium bromide and visualized as UV fluorescence. If the amount of PCR product was considered too small for DNA sequencing (visual estimation of fluorescence), reamplification of the PCR product was performed (I). This reamplification consisted of 18 thermal cycles. The reamplified product was then purified from 1.2 % the low-melt agarose gel (BDH Laboratory Supplies, Poole, England) using QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany).

3.4 Cloning of PCR products (II, III)

Amplified PCR products were ligated into the pCR2.1-TOPO vector and transformed into *E. coli* cells by using the TOPO TA cloning system (Invitrogen, Carlsbad, CA, USA). Two separate clone libraries were created from each PCR product amplified from tissue digest supernatant. Transformants were analyzed by picking colonies and resuspending them individually in 50 µl of PCR cocktail consisting 10 pmol/µl of M13 Forward (-20) and M13 Reverse primers (Table 2), and standard amounts of HotStarTaq Master Mix-reagent containing 2.5 units HotStarTaq DNA Polymerase, 15mM $MgCl_2$, and 200µM of each dNTP (Qiagen). The PCR amplification procedures are described in original communications II and III. Success in ligation of inserts of the expected size was verified by electrophoresis in a 2.0 % LE analytical grade (Promega, Mannheim, Germany) agarose gel containing ethidium bromide and visualized as UV fluorescence. Positive transformants were purified by using QiaQuick PCR Purification Kit (Qiagen GmbH, Hilden, Germany).

3.5 DNA Sequencing

Semi-automated sequencing method was used for sequencing of PCR products. The sequencing primers are shown in Table 2. Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) (I-III) and BigDye Terminator Cycle sequencing chemistry (Applied Biosystems) were used for sequencing reactions. Cyclic sequencing steps (96 °C for 30 seconds, 50 °C for 15 seconds, 60 °C for 4 minutes) were repeated 25 times using a TechGene (Techne, Burlington, USA) (II-III) thermal cycler. Unincorporated dye terminators were then removed by using a DyeEx 2.0 Spin kit (Qiagen GmbH, Hilden, Germany). Sequencing products were resolved using the automated ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

3.6 Sequence analyses

Both DNA strands were analysed and base-editing was performed together with manual review of the electropherograms (I-III). The nucleotide sequences were edited and aligned using the Chromas 2.31 (Technelysium) and ClustalW sequence analysis software (III), and compared with those in the GenBank (Benson *et al.* 2007) database by using the BLAST search tool (Altschul *et al.* 1990) available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Initial alignment of amplified sequences in original communication III was performed using the automated 16S rRNA sequence aligner of the ARB software (Ludwig *et al.* 2004) against a database of 102, 134 complete and partial rRNA sequences. Ambiguously and incorrectly aligned positions were aligned manually on the basis of conserved primary sequence and secondary structure. The phylogenetic associations were determined from 495 masked positions using a maximum-likelihood algorithm (Felsenstein 1981, Olsen *et al.* 1994). These associations were confirmed using a least-square fit (De Soete 1983) of Jukes-Cantor corrected evolutionary distances and maximum parsimony algorithms.

3.7 Arrangement of PCR laboratory and PCR work

To avoid contamination, all steps of PCR analysis (DNA extraction, preparation of PCR mixtures, amplification, cloning, and analysis of PCR products) were performed in separated places. PCR reaction mixtures were prepared in a sterile laminar flow, used only for this purpose. All the PCR reagents and DNA samples were added to the reaction mixture using pipettes with filtered tips. A sample preparation control with sterile water that went through the same DNA extraction steps as the clinical samples served as a negative control. As a positive control, DNA from *Escherichia coli* strain B (ATCC 11303) (Sigma, St.Louis, USA) was used.

4. Men with myocardial infarction from the 1997 FINRISK study (IV)

Every five years since 1972 the National Public Health Institute of Finland has performed large, cross-sectional population surveys related to the risk factors of coronary heart disease in five geographic areas. The present population originated from the 1997 FINRISK study, which had a total sample size of 11 500 subjects: 6000 men, 5500 women. The survey included a self-administered questionnaire that was sent to the subjects in advance including 165 questions about previous and existing diseases, which participants returned to the survey site. Subsequently, the participants' height, weight, and blood pressure were measured using standard procedures, and a venous blood specimen was taken. Body mass index (BMI) was calculated as the ratio of weight (kg) to height squared (m^2). Subjects with a history of previous MI were defined on the basis of the answers. In order to be included in the MI group, the participants needed to have had a MI that was diagnosed by a physician. A group of 120 men with a history of diagnosed MI and 250 age-matched controls were selected from the total sample population evenly from different regions**.** The study was conducted according to the Helsinki Declaration of 1975 on Human Experimentation and was approved by the Ethical Committee of Primary Health Care Clinics in Finland.

5. Lipid analyses (IV)

Total cholesterol and triglycerides were determined from fresh serum samples by an enzymatic method (Roche Diagnostics, F. Hoffmann-La Roche Ltd.). HDL cholesterol was measured with the same enzymatic method after precipitation of LDL and VLDL with polyethylene glycol.

6. Measurement of serum CRP and C3-complement (IV)

Serum CRP and C3 were determined by using *N High Sensitivity CRP* and *N Antisera to Human Complement Factor C3c* -assays as recommended by the manufacturer (Dade Behring, Marburg, Germany). Immunonephelometry was

performed using BN™ Systems (Dade Behring, Marburg, Germany). The concentrations of the samples were determined versus dilutions of standards of known concentrations.

7. Determination of autoantibodies against oxLDL (IV)

Antigens for this assay included native LDL (natLDL) prepared from the pooled plasma of three donors and protected against oxidation by 0.27 mM ethylenediaminetetraacetic acid (EDTA) and 20 μM butylated hydroxytoluene (BHT) in phosphate buffered saline (PBS), and oxLDL obtained after 24-h oxidation of the natLDL with 2 μM $CuSO_4$. An enzyme-linked immunosorbent assay (ELISA) was used to determine autoantibodies against oxLDL. Exact procedure is described in original communication (I). Shortly, conditions of the assay were as follows: coating concentration 5 μg/mL of natLDL and oxLDL on a microtiter plate, blocking with 2% human serum albumin (HSA) in PBS containing 20 μM BHT and 0.27 mM EDTA, sample dilution 1:30, and conjugate dilution 1:4000 in 0.27 mM EDTA, 20 μM BHT, 1 % HSA, 0.05 % Tween in PBS. The optical density (OD) was measured at 492 nm using a microplate reader (Wallac 1420 Victor™, Wallac Oy, Turku, Finland).

8. Determination of serum MMP-9 concentration (IV)

For the accurate assessment of serum MMP-9, blood samples were centrifuged within an hour after venipuncture. Aliquots of sera were removed and stored at -70 °C until analysis in a freezer that was not in daily use. Quantitation of immunoreactive MMP-9 was carried out by ELISA (Diabor Ltd, Oulu, Finland). Recombinant MMP-9 was used as standard. The microtiter plate was coated with the monoclonal antibody (code GE-213). The bound proteins from serum and standards were detected with a secondary polyclonal antibody produced in chicken against MMP-9. A peroxidase-labeled anti-chicken-IgG (Chemicon, USA) was used for detection of the bound secondary antibody. O-phenylenediamine (OPD) was used to visualize the peroxidase label. The color formation was measured at 450 nm (Anhos

2000 microplate reader) and calculations were done using a Multicalc program (Wallac, Finland). The monoclonal antibody recognizes both the free MMP-9 and that bound to its inhibitor, tissue inhibitor of metalloproteinases-1 (TIMP-1).

9. Statistical analysis (IV)

The statistical evaluation was done with a microcomputer using Statistica for Windows version 5.1 (Statsoft, Inc., Tulsa, OK, USA). Values of the MI group and controls were compared by Mann Whitney U-test. Logistic regression analysis was used to evaluate the factors predicting coronary infarction. All results are expressed as mean (±SD). *P*-values less than 0.05 were considered as being statistically significant.

RESULTS

1. Morphological characteristics of study series (I- III)

In the temporal artery sample series (I), all temporal arteritis specimens showed strong medial and adventitial inflammation characterized by numerous macrophages and lymphocytes, whereas normal temporal artery samples showed normal morphology with no inflammation. Nineteen of the 27 temporal arteritis specimens had characteristic infiltrates of giant cells. No bacterial structures were visualized. The lung sample, obtained from the autopsy of a man who died of sepsis, exhibited strong infiltration of polymorphonuclear leukocytes, consistent with an acute infection, but no bacterial structures. See Figure 1A-C in original communication I.

All coronary artery specimens showed different types of atherosclerotic lesions regardless of cause of death (II). Four of the five cases, whose cause of death was coronary artery disease (CAD), had type Va or Vb lesions. In addition, all five control coronary specimens had different types of atherosclerotic lesions from fatty streaks to small atheroma. The abdominal aorta samples exhibited type Va or Vb atherosclerotic lesions (III). In addition, all abdominal aorta specimens exhibited inflammation with variable amouts of leukocyte infiltration. Figures 2A and 2B show an example from an abdominal aorta (unpublished data).

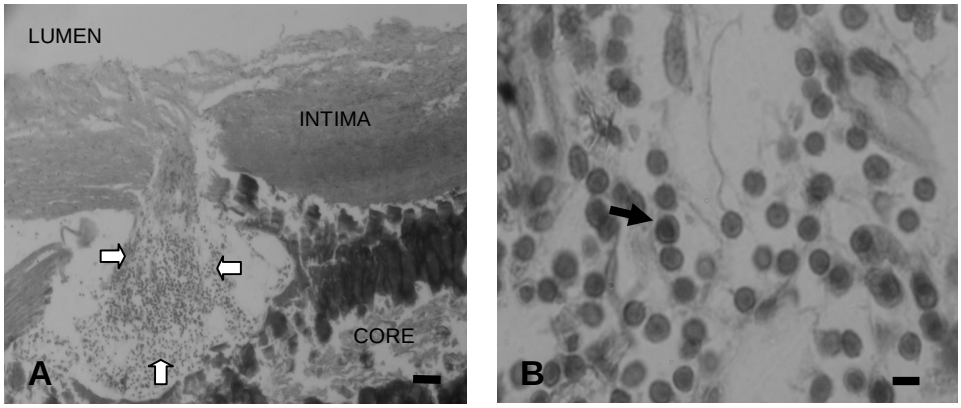

Figure 2. Histologic section from an abdominal aorta fibroatheroma with infiltration of leukocytes (block arrows and arrow); staining with hematoxylin-eosin. Bar in figure 2A is 100 µm and in 2B 10 µm.

2. Bacterial DNA in temporal arteritis (I)

No positive DNA bands of the correct size were visible in any temporal artery specimens by agarose-gel electrophoresis and ethidium bromide staining after 28 cycles of amplification. PCR control reactions without added tissue DNA were also negative. In the lung sample, a product of the expected 290-bp size was visualized. The 16S rDNA sequence similarity of the lung sample was 99,3 % (289/ 291 bp) with *E. coli* sequence. Faint bands of the specific length could be seen in all reaction mixtures after 40 or more cycles of PCR amplification. After sequencing, these 16S rDNA sequences shared 100 % homology with those of *Pseudomonas echinoides* and *Sphingomonas sp.* partial 16S rRNA gene (291 / 291 bp) [PEU96454 and SS16SPC58]. All temporal artery specimen extracts were positive for human β-actin by PCR, indicating that there were no inhibitory components derived from arteries in the PCR reactions and that the DNA in the samples was intact. The sensitivity of the Br-PCR with purified *Escherichia coli* strain B DNA (Sigma, St. Louis, MO) was 5.0 fg of DNA per assay (corresponding to approximately 7 *E. coli* rDNA copies and one *E. coli* genome copy). See Figure 2 in original communication I.

3. Bacterial DNA in human coronary atheromas (II)

Ten coronary arteries from 10 autopsy cases were studied. All of the samples were positive by β-actin PCR. The sequence comparison revealed that all coronary specimens contained DNA sequences of oral microflora. The bacterial species identified after cloning and sequencing are summarized in Table 1 of original communication II. The finding of *Escherichia coli* sequences that were detected both from two coronary arteries and extraction control reagents was omitted as their potential origin as methodological contaminants possibly from *Taq* DNA polymerase (Nikkari *et al.* 2001) could not be ruled out.

4. Bacterial DNA in abdominal aorta atherosclerotic lesions (III)

Of all 20 abdominal aorta samples analyzed, 11 (55%) were positive by the 16S rDNA PCR done with 38 cycles. All the five negative control reactions were negative when analyzed as the clinical samples. In order to rule out methodological contaminants, 5 control samples without tissue extract were given 12 more PCR cycles than the aorta samples (total of 50 cycles) with subsequent cloning and sequencing. A bacterial diversity of over 45 different species was demonstrated in 160 sequences from 11 patient samples (subjects A-K) and the 5 control samples. Mean bacterial diversity in atheromas was then 5.5 ± 1.3. The sequences of positive clinical samples and the sensitized negative controls were included in the systematic phylogenetic analysis. In order to clean out possible background sequence-types deriving from the DNA purification and PCR amplification reagents, the patient bacterial sequence-types that showed over 99% similarity to those from sensitized controls were omitted. After this subtraction procedure, all sequence-types from two initially positive patient samples (subjects G and J) were dropped out from the final results as their potential origin as methodological contaminants could not be ruled out. Finally, the mean number of bacterial sequence-types showing diversity of 99% or less within each group from the remaining 9 patient samples was 2.2 ±1.2, mean length of sequences being 506.6 ± 22.2 bp.

Phylogenetic relationships among the atherosclerosis associated bacterial 16S rDNA sequences, the relatedness of the consensus sequence of each abdominal aorta sequence-type with those available at GenBank, and a total of 29 potential non-relevant sequence-types representing 26 species that were present in the libraries from both abdominal aorta- and negative control PCR products are summarized in original communication III (Figure 1, Table 1, and Table 2, respectively).

5. Inflammatory markers in men with a history of myocardial infarction (IV)

The MI group had lower serum total cholesterol concentrations than controls, but when subjects with cholesterol-lowering medication were excluded, there was no variation in total cholesterol between the groups, even though concentrations of HDL cholesterol remained lower and triglycerides higher in the MI group.

Serum MMP-9 concentration was higher in the MI group than in control subjects ($p= 0.037$, Mann Whitney U-test). Levels of the inflammatory markers CRP and C3 were also higher in the MI group compared to controls ($p= 0.004$, and $p= 0.006$, respectively). When oxLDL titers were determined, no difference was found between the MI group and control subjects as measured by ox-LDL-natLDL (the binding of autoantibodies to natLDL is subtracted from that to oxLDL).

When independent variables (BMI, HDL cholesterol, triglycerides, CRP, C3, and MMP-9) predicting MI were analyzed by stepwise multiple logistic regression, the significant predictors for MI were HDL cholesterol ($p=0.002$) and MMP-9 ($p=0.015$).

DISCUSSION

1. Bacterial DNA in temporal arteritis (I)

Molecular studies of TA have suggested a possible link between infection and TA, in particular *Chlamydia pneumoniae* (Rimenti *et al.* 2000, Wagner *et al*. 2000) and parvovirus B19 (Gabriel *et al*. 1999, Salvarani *et al*. 2002b). In the first study, we chose to investigate temporal artery biopsies for evidence of bacterial involvement in TA by using broad-range 16S rDNA PCR.

We found bacterial DNA neither in 27 temporal arteritis samples nor in five normal temporal artery samples. Our findings are in concordance with study reports where DNA of parvovirus B19, *Chlamydia pneumoniae*, or human herpes virus could not be detected from temporal arteritis specimens (Haugeberg *et al.* 2000, Helweg-Larsen *et al.* 2002, Regan *et al.* 2002, Rodriguez-Pla *et al.* 2004). Inhibitors of the PCR reaction cannot explain our negative results since β-actin could be amplified from all clinical specimens.

Greer and co-workers (Greer *et al.* 1991) have suggested that fresh-frozen tissue would be the preferable specimen for broad-range PCR because formalin fixation can damage nucleic acids at particular positions causing higher polymerase error rates. Yet, we were able to identify *E. coli* in a formalin fixed paraffin-embedded lung sample from a patient with sepsis and bacterial pneumonia indicating that our DNA extraction procedure was adequate. Low amounts of bacteria could have been detected in the temporal arteritis specimens because the sensitivity of our PCR assay was 5.0 fg of DNA. However, the lack of detectable amounts of bacterial rDNA sequences do not rule out the possibility for bacterial degradation products as the inflammatory agents, or of bacterial involvement in triggering an autoimmune host defense mechanism.

2. Bacterial DNA in CAD (II)

Various studies have suggested that diverse bacterial colonization in the atheroma may be more important than a single pathogen in the link between infection and atherosclerosis. Additionally, there is at present ample evidence to suggest that oral (Pussinen *et al.* 2003, Pussinen *et al.* 2005) and other respiratory tract bacteria, such as *Chlamydia pneumoniae* (Linnanmäki *et al.* 1993, Miyashita *et al.* 1998), are strongly associated with coronary disease. Broad-range 16S rDNA PCR was used to evaluate the bacterial flora of coronary arteries from five subjects with sudden coronary death and five controls.

The roles played by the organisms that were detected in coronary artery specimens are open to speculation. First, there is *Streptococcus pyogenes* that was the only bacterial species detected in 67-year-old man coronary artery. The known association of this organism with rheumatic heart disease is well-established (Carapetis *et al.* 2005). Molecular mimicry between pathogen and human proteins has been proposed as a triggering factor leading to autoimmunity in this disease. Evidence suggests that *S. pyogenes* contains proteins that exhibit some degree of homology to host proteins that they can stimulate existing B and T lymphocytes to respond to self proteins (Cunningham 2003). This is known to lead to autoimmune post-streptococcal rheumatic carditis involving the myocardium and valves. Second, there are the oral microbes which have been suspected to enter the blood stream during transient bacteremias. These microbes (See Table 1 in original communication II) are typical bacteria in normal oral flora. They are weak pathogens and and do not generally cause infections in healthy individuals. However, it is possible that they have no role in atherogenesis and act more as "innocent bystanders".

We observed a wide palette of oral bacterial signatures in all coronary specimens. Our findings are in line with a recent study conducted on coronary atherectomy samples, where direct universal bacterial 16S rDNA PCRs gave high overall diversity of bacterial signatures originating from skin, respiratory, or oral flora (Ott *et al.* 2006). We suggest that atheromas may act as mechanical sieves collecting

bacteria from the circulation. Actually, bacterial DNA has been previously reported to be present in blood samples of healthy individuals (Nikkari *et al.* 2001), and it is likely that the oral cavity and other mucosal surfaces, e.g. the gut, represent the most common origin of transient bacteremia. The presence of such pathogens in atherosclerotic lesions may, in certain individuals, contribute to the development of clinically significant CAD.

3. Bacterial DNA in abdominal aorta atherosclerotic lesions (III)

In the third study, we defined bacterial DNA in surgically removed sterile abdominal aorta samples of patients with aortic atherosclerosis by using a novel subtraction Br-PCR approach. Our histochemical investigation showed distinct localization of leukocytes in atherosclerotic lesions obtained from both AAA and ASO patients indicating clear inflammatory process on site (unpublished data). Ample evidence suggests that infectious agents may play a role in the pathogenesis of atherosclerosis and in the clinical manifestations of vascular disease. However, as outlined above, most of the studies have concentrated on the potential role of only one specific microbe, such as *C. pneumoniae* or *H. pylori*. Less frequently, a broader multi-microbial presence in the etiology of the atherosclerotic plaque has been suggested (Watt *et al.* 2003, Ott *et al.* 2006). From abdominal aortas, we were able to detect a wide range of different bacterial DNA signatures. Our results are in concordance with a recently published study by Marques da Silva *et al.* (2006) who hypothesized that bacteria might play role in the inflammatory reaction leading to aneurysm formation. We found bacterial rDNAs also from the aorta wall of patients with ASO, which is a disease consisting of focal or diffuse narrowing or occlusion of peripheral arteries due to atherosclerotic deposition. Atherosclerosis is considered a common underlying etiologic factor for both ASO and AAA (Reed *et al.* 1992).

Our study underlines the necessity that when low DNA template concentrations are used in Br-PCR, elimination of background sequence-types should be performed from the final results. Of note is that bacterial rDNA sequences were not detected from the negative controls of this study when they were prepared and analysed as the clinical samples. However, the reagent controls, when analysed with 12

additional PCR cycles, gave a multitude of low-level bacterial signals that were mostly associated with water- and soil-associated bacterial species. With the purpose of removing a multitude low-level background bacterial signals that was potentially affecting our analysis and interpretation of the sequence-types deriving from the clinical samples, those bacterial sequences from clinical samples showing over 99% similarity sequences from optimized controls were omitted from our analysis. Similar contaminant bacterial sequence-types that were present in our negative control PCR products were reported by Marques da Silva and his colleagues (2006) to be present in aortic aneurysm samples. These included *Bradyrhizobium* sp. and *Comamonas testosteroni.*

The roles played by the bacterial sequence-types that we identified from abdominal aortas in AAA and ASO are at present unknown, but there are previous reports indicating that ten of the identified 16 bacterial species (63%) represent human pathogenic bacteria (See original article III). These included *Staphylococcus hominis* (Sunbul *et al*. 2006, Kessler *et al*. 1998), *Micrococcus luteus* (Albertson *et al*. 1978, Seifert *et al*. 1995) *Corynebacterium* sp. (Khamis *et al*. 2005), *Corynebacterium sundsvallense* (Collins *et al.* 1999), *Corynebacterium tuberculostearicum* (Vedel *et al*. 2006), *Acidovorax* sp. (Shetty *et al.* 2005), *Brevundimonas diminuta* (Paster *et al.* 2002, Han *et al*. 2005, Marques da Silva *et al*. 2006), *Comamonas* sp. (Horowitz *et al.* 1990, Stonecipher *et al.* 1991), *Sphingomonas* sp. (Martino *et al.* 1996, Hsueh *et al.* 1998), and "freshwater bacterium" sharing 98.1 % similarity to a beta proteobacterium sp. [AY005031] sequence detected from a patient with periodontitis (Paster *et al.* 2001).

Four sequence-types were related with bacteria without any published clinical association. The remaining two sequence-types from the clinical samples were not closely related (less than 97% sequence similarity to known sequences) to any published bacterial 16S rDNA sequences submitted to GenBank, suggesting these were potential new species.

The difficulty in detection of *C. pneumoniae* or other more widely suspected atherogenic species by universal bacterial DNA amplification does not necessarily mean that other bacterial species are more common visitors of atherosclerotic lesions, but may be explained by low sensitivity of this assay to these pathogens (Olmez *et al.* 2001). Several studies performed with species-specific primers have reported the presence of *C. pneumoniae* in samples of atherosclerotic arteries (Campbell *et al.* 1995, Ramirez 1996). This indicates that when using specific DNA primers the sensitivity of the PCR may be higher. However, the presence of both commensal and pathogenic bacteria in atherosclerotic lesions suggests that it is unlikely that a single microbe could act as the causative agent behind atherogenesis or plaque rupture.

4. Inflammatory markers in CHD (IV)

A great amount of data indicates that inflammation plays an important role in all stages of atherosclerotic process (Ross 1999a,b). This recognition has inspired the evaluation of diverse inflammatory markers as potential predictors of cardiovascular risk. The purpose of study IV was to compare serum MMP-9, CRP, C3-complement (C3), and autoantibodies against oxLDL in male subjects with a history of previous MI to controls from a cross-sectional population survey, the FINRISK study.

In original communication IV, raised serum concentrations of MMP-9 were found in the MI group. This is in line with our previous observations and those of others that this protease is increased in sera of subjects with severe and complicated coronary heart disease (CHD) (Kalela *et al.* 2002, Fukuda *et al.* 2006). Therefore, we suggest that elevated serum levels of MMP-9 in MI may be associated with arterial inflammation that is related to progression of atherosclerosis. In fact, increased concentrations of MMP-9 have also been reported in asthma (Kelly *et al.* 2000) and rheumatoid arthritis (Gruber *et al.* 1996), which support this association.

To date, CRP has been reported to be increased in CHD by various studies (Biasucci *et al.* 1999, Fichtlscherer *et al.* 2000, Blackburn *et al.* 2001, Burke *et al.* 2002), including our present findings. Components of the complement participate in the

inflammatory process, and CRP modifies and attenuates this response (Ablij *et al.* 2002). Thus, it is not surprising that the most important member of the complement, C3, was elevated in subjects with a history of MI compared to controls. Other studies support these findings (Muscari *et al.* 1995, Muscari *et al.* 1998).

Statins have been shown to have many anti-inflammatory effects (Ablij *et al.* 2002). These include decreasing serum CRP levels in subject with CHD and serum MMP-9 concentration in clinically healthy men which may reflect reduction of chronic arterial inflammation (Kalela *et al.* 2001). Most of the men with a history of MI (38 %) were on statin treatment compared to controls (9 %). The difference in statin treatment might have attenuated some of the differences in serum inflammatory parameters observed in the present study. Additionally, the serum antibody levels against oxLDL were not elevated in subjects with history of MI.

5. A hypothesis of the role of infection in arterial inflammation

Our hypothesis on how bacterial DNA is deposited in arteries is shown in Figure 3. The diversity of bacteria discovered from atherosclerotic arteries suggests that atheromas might act as mechanical sieves collecting bacteria from the circulation. Based on the present findings, we speculate that the microbes may have originally entered the blood stream from various tissues during transient bacteremias known to occur e.g. during toothbrushing or by leaking through mucosal surfaces. The presence of such pathogens may, in certain individuals, contribute to the development of clinically significant atherosclerosis. A series of acute and chronic infections, occurring alone or in combination, may accelerate atherogenesis by increasing inflammation by stimulation of leukocyte activation/infiltration in the atherosclerotic arteries. In fact, systemic markers of such arterial inflammation, e.g. MMP-9, are predictors of atherosclerotic events (Sorbi *et al.* 1996, Koenig *et al.* 1999). On the other hand, bacterial DNA may also enter the bloodstream and subsequently the arterial wall from all substances ingested since it is potentially present everywhere.

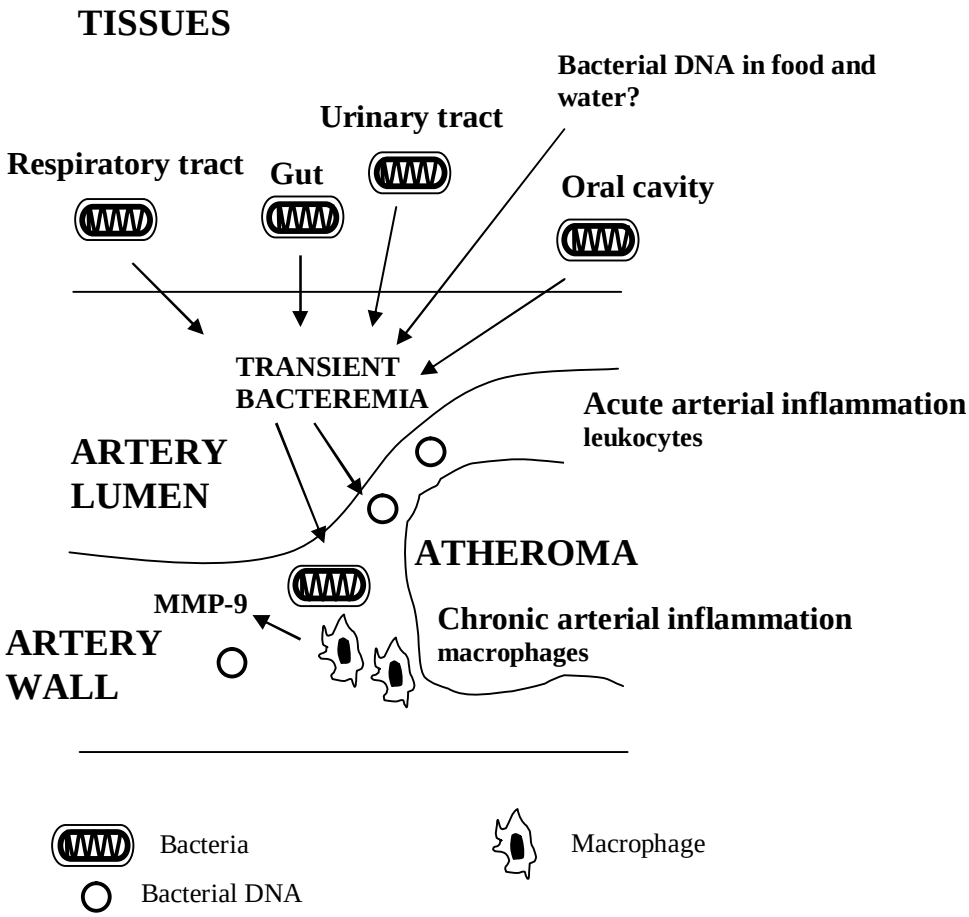

Figure 3. Hypothetical mechanisms of infection in arterial inflammation.

SUMMARY AND CONCLUSIONS

Inflammation has been shown to play an important role in the clinical complications of atherosclerosis. This is mediated partly through upregulation of proteolytic enzymes by leukocytes. Bacterial infections are potential factors that may induce and promote inflammatory process. The present study investigated whether bacterial DNA is present in arterial walls of different clinical specimens by using broad-range 16S rDNA PCR methodology. In addition, serum MMP-9 and other inflammatory markers in male subjects with a history of previous MI were compared to controls. The findings and conclusions are:

1. The lack of detectable amounts of bacterial DNA from temporal arteritis samples suggests that viable bacteria do not have a role in chronic stages of this disease. However, these findings do not exclude the possibility of bacterial degradation products as stimulants of chronic inflammation, or of viable microbes as triggering factors of acute temporal arteritis.

2. The high overall diversity of bacterial DNA in atherosclerotic coronary and abdominal artery specimens suggests a multi-infection role of microbes in the pathogenesis of atherosclerosis.

3. Elimination of background sequence-types should be performed from the final results of Br-PCR. Therefore, we created a novel subtraction analysis to exclude background signal.

4. Elevated serum MMP-9 concentration in subjects with a history of MI may reflect inflammatory pathologic processes that are related to progression of atherosclerosis.

Generally, it can be concluded that atheromas may act as mechanical sieves collecting bacteria from the circulation. The subsequent arterial infection may enhance arterial inflammation and thus the progression of atherosclerosis.

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ORIGINAL COMMUNICATIONS

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Do temporal arteritis lesions contain bacterial DNA?

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Abstract

Background Temporal arteritis is a primary vascular inflammatory disease. The aetiology of temporal arteritis is unknown, but the influence of environmental factors such as infections has been suggested.

Materials and methods We used broad-range PCR, targeting conserved regions of the gene encoding for ribosomal RNA, to detect bacterial DNA in 27 temporal artery biopsies. Five uninvolved temporal arteries were also included. A lung sample of confirmed bacterial pneumonia served as a positive control. Inflammation was examined by histochemistry and light microscopy.

Results The sensitivity of the broad-range PCR assay was 5·0 fg of DNA. Bacterial DNA sequences were neither detected in 27 temporal arteritis specimens nor in the normal temporal artery samples. However, bacterial DNA was successfully amplified from the lung sample of a subject with pneumonia. In addition, human DNA was amplified by primers for human β-actin from all clinical specimens, suggesting lack of significant inhibitors of the molecular amplification reaction. Histochemistry showed signs of strong inflammation in the arteritis samples.

Conclusions The lack of detectable amounts of bacterial DNA suggests that viable bacteria do not have a role in chronic stages of temporal arteritis. However, these findings do not rule out the possibility of bacterial degradation products as stimulants of chronic inflammation, or of viable microbes as triggering factors of acute temporal arteritis.

Keywords Bacterial infections, bacterial, DNA, PCR, temporal arteritis/microbiology, temporal arteritis/pathology.
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Introduction

Temporal arteritis is a primary vasculitis of unknown aetiology that affects large- and medium-sized arteries. This disease has a marked female predominance and restriction to old age. It has a high incidence in populations with Scandinavian lineage, some familial accumulation, and an association with the HLA-DR4 haplotype, which indicate a genetic predisposition [1]. Many studies on temporal arteritis biopsies have suggested that this vasculitis might be triggered by infection [2–4], but direct evidence of an infectious cause is still lacking. Epidemiological observations have also suggested an infectious origin [5–7], but temporal arteritis has not been shown to be a truly infectious form of vasculitis to date [8].

Immunological research indicates that temporal arteritis is an antigen-driven disease with local T-cell and macrophage activation. Subsequently, macrophages appear to destroy the internal elastic membrane. This process leads to extensive intimal thickening and causes arterial stenosis and ischaemic complications. The production of cytokines and growth factors by the inflammatory cells possibly contributes to the formation of the neointima [1].

Broad-range bacterial PCR is based on the use of primers that recognize conserved sequences of bacterial chromosomal genes encoding ribosomal RNA (rDNA) [9,10]. The resulting amplified rDNA sequences also include variable regions that provide an alternative approach for identifying

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theoretically all bacterial species, including those that cannot be cultivated by classical methods. We used this methodology for 'molecular mining' of bacterial rDNA sequences from temporal arteritis lesions to identify possible bacterial pathogens as aetiopathogenetic agents of this chronic inflammatory disease.

Materials and methods

Patients and histochemistry

Temporal artery specimens were obtained from 27 patients with temporal arteritis (20 women, seven men; mean age 75 years; range 61–99 years). The disease was diagnosed on the basis of classical clinical and histological criteria [11]. Five uninvolved temporal arteries were also included (four women, one man; mean age 64 years; range 54–80 years). Tissues were fixed in 10% formalin. After paraffin-embedding, transverse sections were cut and used for histochemistry and DNA extraction. For microscopy, the samples were stained with haematoxylin-eosin. In order to ascertain that the broad-range PCR for bacterial rDNA can be utilized for formalin-fixed paraffin-embedded tissue samples, a lung sample was obtained from a 91-year-old man who died of sepsis and bacterial pneumonia following gastrointestinal surgery. This sample was fixed and treated the same way as the temporal artery specimens and acted as a positive control. The study protocol was approved by the Ethics Committee of Tampere University Hospital.

DNA extraction

Paraffin-embedded transverse sections of temporal arteries and lung sample were deparaffinized with xylene and ethanol. Tissues were digested with proteinase K (Finnzymes, Espoo, Finland) at 56 °C for 3 h. After heat inactivation of proteinase K, the reaction mixture was centrifuged and the supernatant was used for PCR analysis [12].

Oligonucleotide primers

The broad-range bacterial 16S rDNA oligonucleotide primers p516F (5′-TGC CAG CAG CCG CGG TAA-3′) and p806R (5′-GGA CTA CCA GGG TAT CTAAT-3′), and the primers for human β-actin BAKTIN-1 (5′-TGA CTG ACT ACC TCA TGA AGA TCC TCA CCG-3′) and BAKTIN-2 (5′-CCA CGT CAC ACT TCA TGA TGG AGT TG-3′) were synthesized at the DNA Synthesis and Sequencing Laboratory, Biocentre Helsinki, Finland.

PCR amplification

Each PCR reaction contained 5 μL of the supernatant from DNA extraction, 20 pmol μL^{-1} of each primer, and standard amounts of HotStarTaq Master Mix-reagent (Qiagen GmbH, Hilden, Germany) in a 50-μL reaction volume. Success in DNA extraction from clinical specimens was verified by the ability to amplify a 290-bp human β-actin gene sequence.

After activation of Taq-polymerase for 15 min at 95 °C, 28 cycles of amplification with broad-range bacterial rDNA primers were carried out in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Each cycle consisted of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 1 min of extension at 72 °C. Last cycle was followed by final extension step at 72 °C for 10 min. Every set of amplification reactions included extraction control reactions without added tissue extract. DNA extracted from the lung sample of a male who died of sepsis served as positive control. For human β-actin, 35 cycles of amplification with primers BAKTIN-1 and BAKTIN-2 were carried out after activation of Taq-polymerase for 15 min at 95 °C. Each cycle consisted of 40 s of denaturation at 94 °C, 1 min of annealing at 69 °C, and 2 min of extension at 74 °C. The last cycle was followed by a final extension step at 72 °C for 10 min. Amplification products were separated by electrophoresis in a 1·5% agarose gel containing ethidium bromide and visualized as UV fluorescence.

Purification and sequencing of the PCR products

For sequencing, the PCR products were reamplified. The reamplification consisted of 18 cycles and was performed as described earlier. The reamplified PCR products were purified from the low-melt agarose gel (BDH Laboratory Supplies, Poole, UK) using QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and sequenced with a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an automated sequencing system (Applied Biosystems DNA Sequencing System).

Results

All 27 temporal arteritis specimens exhibited strong chronic medial and adventitial inflammation, characterized by numerous macrophages and lymphocytes, but only occasional neutrophils. Nineteen of the 27 temporal arteritis specimens had characteristic infiltrates of giant cells. The artery lumen was severely narrowed by intimal hyperplasia (Fig. 1a). The uninvolved temporal artery specimens showed normal morphology, with no inflammation. No bacterial structures were visualized. The lung sample obtained from the autopsy of a man who died of sepsis following gastrointestinal surgery showed strong infiltration of polymorphonuclear leucocytes, consistent with an acute infection, but no bacterial structures (Fig. 1d).

When purified *Escherichia coli* strain B DNA (Sigma, St. Louis, MO) was used as the template in a serial dilution, the sensitivity of the PCR with broad-range bacterial primers p516F and p806R was 5·0 fg of DNA per assay

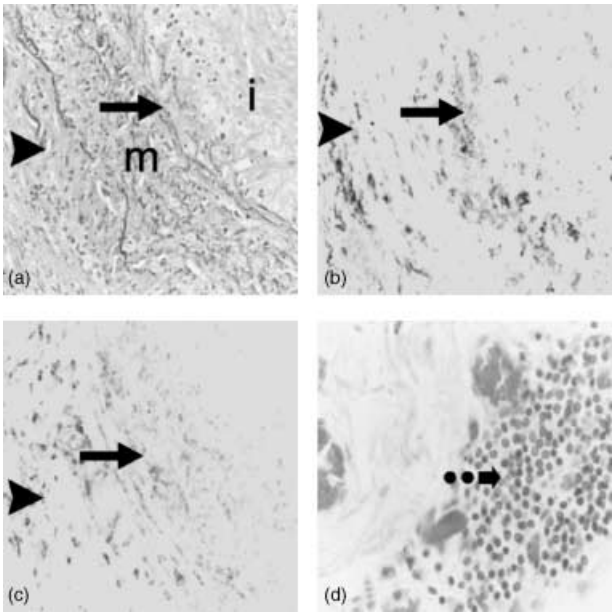

Figure 1 (a–c) Histologic cross sections of inflamed temporal arteritis. Arrows point to the intima-media boundary, arrowheads show the boundary between media and adventitia and have been placed at the same location in adjacent sections. Nearly occluded lumen is to the right; i, intima; m, media. (a) Temporal arteritis with severe intimal thickening, chronic intimal and medial inflammation; staining with haematoxylin-eosin. (b) Identification of macrophages by immunostaining with antibodies to HAM-56. (c) Immunostaining for T lymphocytes. (d) Histologic section of lung tissue with infiltration of polymorphonuclear leucocytes (dotted arrow); staining with haematoxylin-eosin. Original magnifications in Figure 1 (a–c) ×200 and in (d) ×400.

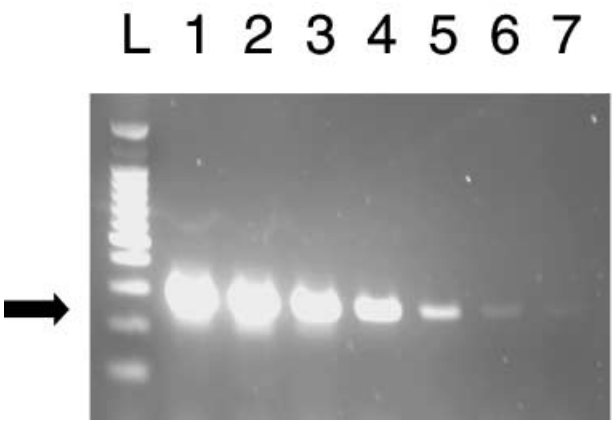

Figure 2 Sensitivity of the bacterial PCR. A dilution series of *Escherichia coli* DNA was analyzed by broad-range bacterial rDNA PCR (28 cycles of amplification) and gel electrophoresis. The arrow indicates the band of the expected size. Lane L, 100-bp DNA ladder (Promega GmbH, Mannheim, Germany); *E. coli* strain B DNA (Sigma, St. Louis, MO) was used in the PCR in amounts of 5·0 ng (lane 1), 500 pg (lane 2), 50 pg (lane 3), 5·0 pg (lane 4), 500 fg (lane 5), 50 fg (lane 6) and 5·0 fg (lane 7).

(corresponding to approximately seven *E. coli* rDNA copies and one *E. coli* genome copy), as visualized after agarose gel electrophoresis and ethidium bromide staining (Fig. 2).

After 28 cycles of amplification using 27 processed temporal artery specimen DNAs as template, no DNA bands of the correct size were visible by agarose-gel electrophoresis and ethidium bromide staining. In the lung sample a product of the anticipated 290-bp size was detected. The 16S rDNA sequence of the lung sample had 99·3% (289/291 bp) similarity with that of six *E. coli* sequences submitted to GenBank [accession numbers AF527827, AF527826, AF527825, AY043392, AF403733, AY082448]. The uninvolved temporal artery specimens and PCR control reactions without added tissue DNA were negative. After 40 or more cycles of PCR amplification, faint bands of the specific length could be visualized in all reaction mixtures. The 16S rDNA sequences from these shared 100% homology with those of *Pseudomonas echinoides* and *Sphingomonas* sp. partial 16S rRNA gene (291/291 bp) [PEU96454 and SS16SPC58]; *Pseudomonas* [13,14] and *Sphingomonas* [15] are common water-associated organisms and known contaminants of PCR reagents [16]. All specimen extracts were positive by human β-actin PCR.

Discussion

Infectious agents have been suggested as aetiologic agents of temporal arteritis. Some studies have indicated that viral infection may play a role in the pathogenesis of temporal arteritis, as demonstrated by studies using molecular amplification with specific primers for parvovirus [2,17] and varicella zoster virus [18]. Therefore, we used broad-range PCR as a means of 'molecular mining' to search for evidence for bacterial involvement in temporal arteritis. Previously, this technique has been shown to provide an effective, non-culture dependent means for detection and identification of bacterial species [9,19].

In contrast to two recent reports, in which *Chlamydia pneumoniae* was detected in tissue samples of temporal arteritis by using touchdown nested [3] and representative PCRs [4] with specific primers, we detected bacterial DNA neither in 27 temporal artery specimens nor in five normal temporal artery specimens. Our results agree with a recent report, in which 180 temporal artery specimens were examined with PCR using established primers for *C. pneumoniae* [20], and chlamydial DNA was not present. Inhibitors of the PCR reaction cannot explain our negative results, as β-actin could be amplified from all clinical specimens.

Fresh-frozen tissue is suggested to be preferable for broad-range PCR, because formalin fixation can damage nucleic acids at particular positions causing higher polymerase error rates [21]. However, in our study *E. coli* was identified in a lung sample from a patient with sepsis and bacterial pneumonia. This finding together with successful β-actin amplification experiments suggests that our DNA extraction procedure was adequate. Furthermore, the same procedure was recently used to amplify *Cardiobacterium*

hominis sequences from a patient with endocarditis [12]. The sensitivity of our PCR assay was 5·0 fg of DNA, which means that low amounts of bacteria could have been detected in the temporal arteritis specimens.

Many studies performed with specific primers for bacteria have reported the presence of *C. pneumoniae* in samples of inflamed and atherosclerotic arteries [3,4,22,23]. Chlamydia have been suggested as aetiologic agents of several other chronic inflammatory diseases as well. In several studies *C. trachomatis* DNA has been detected from joint tissue and synovial fluid of patients with reactive arthritis. Lately, similar findings have been made also from patients with rheumatoid arthritis, and even in joints of apparently normal individuals. In a recent study, *C. trachomatis* and *C. pneumoniae* DNA was detected by nested PCR in joint material from patients with osteoarthitis. However, in that study attempts failed to identify Chlamydial sequences from the joint materials with the broad-range bacterial rDNA approach [24]. This indicates that when using specific DNA primers the sensitivity of the PCR may be higher.

In conclusion, detectable amounts of bacterial rDNA sequences were not present in temporal arteritis lesions, suggesting lack of viable bacteria at the site of inflammation. However, as previously shown in studies on *Salmonella* and *Yersinia*-triggered reactive arthritis [25,26], these findings do not rule out the possibility for bacterial degradation products as the inflammatory agents, or of bacterial involvement in triggering an autoimmune host defense mechanism.

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Identification of different bacterial DNAs in human coronary arteries

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Abstract

Background Various studies have suggested a link between infection, atherosclerosis and coronary artery disease. We studied whether bacterial DNA is present in coronary specimens obtained from left anterior descending coronary arteries of subjects having sudden deaths of cardiovascular and other causes, as verified by an autopsy.

Materials and methods Coronary specimens were obtained from five subjects who died of sudden coronary causes and five controls. Broad-range 16-s rDNA PCR (Br-PCR) amplification, cloning and sequencing were used to detect bacterial rDNA.

Results Bacterial rDNA sequences of oral pathogens were detected from the coronary samples in all cases regardless of the cause of death.

Conclusions Br-PCR is a powerful method to detect bacterial rDNA. By this method we were able to detect wide palette of oral bacteria from coronary tissues. Our findings suggest that atheromas may act as mechanical sieves collecting bacteria from the circulation.

Keywords Bacterial infections, bacterial, coronary artery disease, DNA, PCR.
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Introduction

Inflammation is an important defense mechanism against infection. Chronic or recurrent infections may cause sustained inflammation, which has been linked to increased risk of coronary artery disease (CAD) [1,2]. Several studies have suggested a link between bacterial infection and CAD.

We used a comprehensive PCR methodology to study whether more than one bacterial species might be found in coronary artery tissue material from subjects with CAD. The broad-range bacterial PCR methodology is based on the use of primers that recognize conserved sequences of bacterial chromosomal genes encoding ribosomal RNA (rDNA) [3,4]. The resulting amplified rDNA sequences include variable regions that provide sequence information for phylogenetic identifying of theoretically all bacterial species, including those that cannot be cultivated by classical methods. We used this methodology for 'molecular mining' of bacterial rDNA sequences from coronary lesions obtained from rapid medicolegal autopsies to identify potential aetiopathogenetic agents of this chronic inflammatory disease.

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Materials and methods

Patients and histochemistry

Coronary specimens were obtained from the proximal part of the main trunk of the left anterior descending coronary artery (LAD) of 10 cases within 3 days after death. At autopsy, the proximal part of the LAD was removed and immediately frozen at −70 °C. For histochemistry, the tissues were fixed in 10% formalin, embedded in paraffin, cut and stained with Hematoxyline-Eosine.

This study was approved by Ethics Committee of the National Authority for Medicolegal Affairs, Helsinki, Finland.

DNA extraction

Coronary artery tissues adjacent to the cryostat sections were digested with proteinase K (Finnzymes, Espoo, Finland) at 56 °C for 3 h. After heat inactivation of proteinase K, the reaction mixture was centrifuged and the supernatant was used for PCR analysis [5]. Control extractions of water were also used as a negative control.

PCR amplification

Each PCR reaction contained 5 μL of the supernatant from the DNA extraction, 20 pmol μL^{-1} of each primer, and standard amounts of HotStarTaq Master Mix-reagent (Qiagen GmbH, Hilden, Germany) in a 50-μL reaction volume. Success in DNA extraction from clinical specimens was verified by the ability to amplify a 290-bp human β-actin gene sequence.

Following activation of Taq-polymerase for 15 min at 95 °C, 38 cycles of amplification (94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min) with broad-range bacterial 16-s rDNA primers p516F and p806R [4] were carried out in Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). The last cycle was followed by a final extension step at 72 °C for 10 min. Every set of amplification reactions included extraction control reactions without an added tissue extract. *Escherichia coli* strain B DNA (Sigma, St. Louis, MO) served as a positive control. For human β-actin, 40 cycles of amplification (94 °C for 35 s, 60 °C for 2 min and 72 °C for 2 min) with primers BAKTIN-1 (5′-TGA CTG ACT ACC TCA TGA AGA TCC TCA CCG-3′) and BAKTIN-2 (5′-CCA CGT CAC ACT TCA TGA TGG AGT TG-3′) were carried out after activation of Taq-polymerase for 15 min at 95 °C. Amplification products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide, and visualized as UV fluorescence.

Cloning and sequencing of the PCR products

Amplified PCR products were ligated into the pCR2·1-TOPO vector and transformed into *E. coli* cells by using the TOPO TA cloning system (Invitrogen, Carlsbad, CA). Two separate clone libraries were created from each PCR product amplified from the tissue digest supernatant. M13 forward (–20) and M13 reverse primers were used to analyze transformants by picking colonies and resuspending them individually in 50 μL of PCR cocktail consisting of 10 pmol μL^{-1} of each primer and standard amounts of HotStarTaq Master Mix-reagent. After activation of Taq-polymerase for 15 min at 95 °C, 40 cycles of amplification (95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min) with M13 primers were carried out in an Eppendorf Mastercycler Gradient. The last cycle was followed by final extension step at 72 °C for 10 min. Success in ligation of inserts of the expected size was verified by electrophoresis in a 2% agarose gel containing ethidium bromide and visualized as UV fluorescence. Positive transformants were purified by using QiaQuick PCR Purification Kit (Qiagen GmbH, Hilden, Germany).

After cloning, the automated ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA) and BigDye Terminator Cycle sequencing chemistry (Applied Biosystems) were used to determine the DNA sequences. The consensus sequence from both DNA strands was compared with those in GenBank by the BLAST search tool [6].

Results

Background characteristics of the subjects' coronary artery, morphological findings and PCR results are summarized in Table 1. The bacterial 16S rDNA sequences were 99–100% similar to those submitted to GenBank, including bacterial species belonging to the oral microflora marked in bold. The finding of *E. coli* sequences from two coronary arteries was omitted, as they were owing to Taq DNA polymerase contamination with *E. coli* DNA detected from extraction control reagents [7]. These contaminants become well-represented in recombinant bacterial 16S rDNA clone libraries when studying low concentrations of bacterial DNA specimens [8,9]. All specimen extracts were positive by human β-actin PCR, indicating that the DNA extraction procedure was adequate and that inhibitors of the PCR reaction were not present.

It is noteworthy that all coronary specimens contained DNA sequences of oral microflora. These findings included a common pathogen *Streptococcus pyogenes* and species such as *Actinomyces odontolyticus*, *Haemophilus parahaemolyticus*, *Lactobacillus crispatus*, *Lactobacillus delbrueckii* and *Lactobacillus fermentum*. Additionally, other oral bacteria sequence-types could be identified to the genus level, such as *Lactobacillus* sp., *Neisseria* sp., *Prevotella* sp., *Streptococcus mitis* group, *Streptococcus salivarius* group and *Micrococcus* sp. The species *Lactococcus lactis* sp. and *Enterococcus* sp. are generally regarded as belonging to the gastrointestinal tract flora.

Discussion

Infectious agents have been suggested as aetiologic agents of complicated CAD. We used broad-range PCR as a means of 'molecular mining' to search for the presence of bacterial nucleic acids in autopsy samples of coronary arteries from subjects with sudden death. Use of this technique to identify post mortem coronary artery material has not been reported to date. Previously, this technique has been shown to provide an effective, nonculture-dependent means for detection and identification of bacterial species [3,10].

The roles played by the organisms corresponding to the sequence-types identified in the coronary artery specimens

Table 1 Characteristics of the autopsy cases, coronary artery morphological findings and broad-range bacterial 16S rDNA PCR results

Age	Sex	Cause of death	Morphological findings	Bacterial sequence type	Similarity to GenBank sequences	GenBank accession numbers
79	F	Coronary artery disease	Calcified fibroatheroma	***Neisseria* sp.**	99·3% (289/291 bp)	AY005026 & AJ239301
				***Streptococcus mitis* group**	100% (290/290 bp)	AB006127 & AF385525
				Enterococcus sp.	100% (291/291 bp)	AY172570 & AJ420799
				Lactococcus lactis sp.	100% (290/290 bp)	AF515224 & AE006288
73	M	Coronary artery disease	Calcified fibroatheroma	***Haemophilus parahaemolyticus***	100% (291/291 bp)	AJ295746
67	M	Coronary artery disease	Calcified fibroatheroma	***Streptococcus pyogenes***	100% (290/290 bp)	AE014168 & AE006615
69	M	Coronary artery disease	Fibroatheroma	***Streptococcus salivarius* group**	100% (290/290 bp)	X68418
				***Streptococcus mitis* group**	100% (290/290 bp)	AB006127 & AF385525
				***Prevotella* sp.**		AF385551
56	M	Coronary artery disease	Intimal thickening	***Lactobacillus delbrueckii***	99·3% (289/291 bp)	AY050171 & AB007908
				Lactobacillus fermentum	100% (291/291 bp)	AF243166 & AF243149
33	M	Suicide	Small atheroma	***Lactobacillus delbrueckii***	99·3% (289/291 bp)	AY050171 & AB007908
26	M	Suicide	Intimal thickening; fatty streaks	***Streptococcus salivarius* group**	99·6% (289/290 bp)	X68418
				***Lactobacillus* sp.**	100% (291/291 bp)	AF243173 & AF243160
				Actinomyces odontolyticus	100% (291/291 bp)	AF287751 & AJ234042
35	M	Suicide	Intimal thickening; fatty streaks	***Lactobacillus crispatus***	99·6% (290/291 bp)	AY335503
27	M	Drowning	Intimal thickening; fatty streaks	***Actinomyces odontolyticus***	100% (291/291 bp)	AJ234041
48	F	Pneumonia	Occasional foam cells	***Micrococcus* sp.**	100% (291/291 bp)	AF542073 & AY159889

F, female; M, male; bp, basepairs.
Oral microbes are marked in bold.

is not currently known. We speculate that the microbes may have entered the blood stream during transient bacteraemias known to occur, e.g. during toothbrushing or by leaking through mucosal surfaces. Except for *Streptococcus pyogenes*, most have low virulence and they do not generally cause infections in healthy individuals. However, the microbes may also act as 'innocent bystanders' entering the atheroma.

At present there is ample evidence to suggest that oral [11] and other respiratory tract bacteria such as *Chlamydia pneumoniae* [12] are strongly associated with coronary disease. Br-PCR is powerful method to detect bacterial DNA. Our findings suggest that atheromas may act as mechanical sieves collecting bacteria from the circulation. In fact, bacterial DNA has been previously reported to be present in blood samples of healthy individuals [7], and it is likely that the oral cavity and other mucosal surfaces, e.g. the gut, represent the most common origin of transient bacteraemia. The presence of such pathogens may, in certain individuals, contribute to the development of clinically significant CAD.

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