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Abstract:

Background—Infectious agents, especially bacteria and their components originating from the oral cavity or respiratory tract have been suggested to contribute to inflammation in the coronary plaque, leading to rupture and the subsequent development of coronary thrombus. We aimed to measure bacterial DNA in thrombus aspirates of patients with ST-elevation myocardial infarction (MI) and to check for possible association between bacteria findings and oral pathology in the same cohort.

Methods and Results—Thrombus aspirates and arterial blood from patients with ST-elevation MI undergoing primary percutaneous coronary intervention (n=101, males 76%, mean age 63.3 years) were analyzed with real-time quantitative PCR with specific primers and probes to detect bacterial DNA from several oral species and *Chlamydia pneumoniae*. Median value for total amount of bacterial DNA in thrombi was 16 -times higher than that found in their blood samples. Bacterial DNA typical for endodontic infection, mainly oral viridans streptococci, was measured in 78.2% of thrombi and periodontal pathogens in 34.7%. Bacteria-like structures were detected by transmission electron microscopy in all 9/9 thrombi samples analyzed and whole bacteria in 3/9 cases. Monocyte/macrophage markers for bacteria recognition (CD14) and inflammation (CD68) were detected in thrombi (8/8) by immunohistochemistry. Among the subgroup of 30 MI patients examined by panoramic tomography, a significant association between the presence of periapical abscesses and oral viridans streptococci DNA positive thrombi was found (OR 13.2, 95% CI 2.11 – 82.5; p=0.004).

Conclusions—Dental infection and oral bacteria, especially viridans streptococci may be associated with the development of acute coronary thrombosis.

Key words: thrombus, myocardial infarction, oral bacteria, viridans streptococci, inflammation

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Introduction

Bacterial infections have been suggested to have a role in the etiology of atherosclerosis. Of the infective agents, *Chlamydia pneumoniae* has been the most common suspect (reviewed in [1]), but antimicrobial treatment against *Chl. pneumoniae* failed to show any benefit in the secondary prevention of coronary events[2]. Oral pathogens such as viridans group streptococci and the periodontal pathogen *Porphyromonas gingivalis* have been detected by immunohistochemistry in human carotid atherosclerotic plaques[3]. Using various PCR techniques, periodontal bacterial DNA from *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Treponema denticola,* and *Campylobacter rectus* have been detected in the atherosclerotic lesions of aortic tissues [4-7]. We have previously reported the presence of ribosomal DNA from oral streptococcal bacteria including viridans *Streptococcus mitis* –group[8] in coronary atherosclerotic plaques. Subsequently, other groups have confirmed this finding and found more than 50 different bacteria, including other *Streptococcus* species existing within the same atherosclerotic lesion[9, 10]. Koren *et al.* identified *Chryseomonas* sp. in all, and *Veillonella* sp. and *Streptococcus* sp. in the majority of atherosclerotic plaque samples, and their abundance in plaques also correlated with their abundance in the oral cavity[11]. At present, however, there is no consensus about the most pathogenically relevant bacteria.

Poor dental health has been linked to an increased risk of cardiovascular diseases[12]. Chronic periodontal infection especially has been associated with the risk of acute myocardial infarction (MI)[13]and coronary heart disease[14]. In addition, Mendez *et al.* [15] found that periodontal disease was a significant independent risk factor for peripheral vascular disease. Almost all the patients in their study (51 of 53 [96%]) had moderate or severe periodontitis or were edentulous. The most recent meta-analysis showed an increased risk of the simultaneous presence of

cardiovascular disease in patients with periodontitis [16]. According to a recent large population study, angiographic coronary stenosis is linked to alveolar bone loss, pathological periodontal pockets and the number of missing teeth [17].

In a recent study by Ohki *et al*., periodontal pathogens were detected in the thrombus aspirates of MI patients [18]. Most studies [13-18] have focused to the role of periodontal pathogens and / or periodontal infections as a risk factor for atherosclerosis. We hypothesized that not only typical periodontal bacterial DNA but also other oral bacterial DNA can be detected in the thrombus aspirates of MI patients, while dental procedures are also linked to bacterial findings in the atherosclerotic tissues. We collected a series of thrombus aspirates from ST-elevation MI patients from two heart centers and measured candidate bacterial DNA in these using real-time quantitative PCR. We also aimed to verify the link between periapical abscesses and MI by analyzing the association between bacterial findings and dental pathology in a subset of our patients. To evaluate the pathological significance of our bacterial findings, monocyte/macrophage markers for bacteria recognition (CD14) and inflammation (CD68) were immunohistochemically stained in available thrombus aspirates.

Methods

Subjects and sampling

Myocardial infarction (MI) patients treated with primary percutaneous coronary intervention and clinical parameters

One hundred and -one consecutive patients with acute myocardial infarction (MI) treated with primary percutaneous coronary intervention (PCI) and successful thrombus aspirations were recruited from Satakunta Central Hospital, Pori, Finland and Turku University Hospital, Turku,

Finland between December 2009 and March 2011. Aspiration of thrombi from the culprit artery was performed as a part of the routine treatment of MI in a standardized fashion with sterile equipment (Export Catheter, Medtronic or Quickcat Catheter, Spectranetics). After aspiration, the content of the aspiration syringe was emptied into a pyrogen-free eppendorf tube. A control blood sample was obtained for each patient from the arterial sheath before the procedure and stored in a similar tube to be used as a reference. The PCI procedure was otherwise carried out according to local standards and the sampling of aspirates did not affect the patients' treatment. For clinical laboratory measurements peripheral venous blood samples were collected (non-fasting). Triglycerides, LDL, HDL, and total cholesterol were determined in serum using standard laboratory techniques.

Scoring of dental status

Thirty of 101 the patients (29.5%) were subjected to dental panoramic tomography. Dental pathology was performed in one heart center and included 50 patients. Of those 50 patients, the first 30 subjects were included. With this technique an image of the entire dentition is obtained on a single film or on a digital phosphor plate. The tube and the cassette holder of the panoramic tomography apparatus rotate in a scanning manner from one side of the patient's head around his back to the other side. During this motion the film / plate cassette moves synchronically producing a two-dimensional image of the upper and lower jaws with the orthogonal projections of the teeth (Figure 1). Panoramic x-rays were assessed by a board certified dentist without knowledge of the clinical patient data. For every x-ray picture, 9 parameters of dental findings were scored. Altogether 270 values were assessed for the 30 panoramic radiographs.

We assessed periodontal health as described in detail earlier[19]. Numbers of dental osseous lesions (periapical lesions at the root tips ∅1-4mm), and signs of dental caries (caries lesions

Black's classes I - VI, and residual roots *e.g.* totally decayed teeth) were calculated, and pericoronal lesions (infected areas around 8 th molars) were recorded. Moreover, signs of dental treatments, *e.g.* number of fillings and root canal treatments were recorded. Periodontal health was assessed by scoring vertical bony pockets (depth >3mm) and furcation lesions (III grade *e.g.* no jaw bone left at the base of the root trunk of a tooth where two or more roots meet[20]). Using digital panoramic x-rays scoring was done on a pc workstation (CliniView 7.1, Instrumentarium Dental, USA) and distance / diameter evaluations using panoramic tomography software tools (Carestream, Carestream Health, Canada).

Detection of bacteria

Real time quantitative PCR

Oligonucleotide primers and probes for real time quantitative PCR (qPCR) are listed in Supplementary Table 1. The primers and probes were designed and confirmed using BLAST with the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov) and/or Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp). Amplification primers / probes designed by other laboratories were synthesized according to the sequences published by the authors and examined in the same manner. Detailed information is described in Supplement 1. Briefly, the presence and amount of human DNA, total bacterial DNA, candidate bacterial DNA for endodontic bacteria (*Streptococcus sp.* mainly *Str. mitis*-group, *Str. mitis, Str. oralis, Str. sanguinis & Str. gordonii, Streptococcus anginosus* -group, *Staphylococcus aureus, Staphylococcus epidermidis, Parvimonas micra* and *Prevotella intermedia)* and periodontal bacteria (*Porphyromonas gingivalis, Aggregatibacter (néé Actinobacillus) actinomycetemcomitans, Fusobacterium nucleatum, Dialister pneumosintes,* and *Treponema denticola)* as well as *Chlamydia pneumoniae* were determined in thrombus

aspirates and control arterial blood samples using real time quantitative PCR (RT-qPCR) and the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, Calif.,USA). The relative amounts of bacterial DNA in thrombus were calculated in comparison to that in inner control (blood) using the comparative *Ct* method ($\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{inner control}}$, Suzuki et al 2005)[21] with a simplification. The method gives n-fold difference in the amounts of candidate bacteria between sample and control in relation to a reference gene. Thrombi results were classified into candidate bacteria positive or bacteria negative. The samples were marked to be positive for candidate bacteria if $2^{-\Delta\Delta Ct} >= 2$ or if there was amplified bacterial DNA in the thrombi but not in the control (blood) sample. The bacterial results from the first 11 aspirates were also verified with the Br-PCR as described earlier[8].

Transmission electron microscopy

Randomly selected frozen thrombus aspirates (n=9) for EM were allowed to melt overnight in a glutaraldehyde - formaldehyde mixture (1% glutaraldehyde and 4% formaldehyde in 0.1 mol/liter phosphate buffer, pH 7.4), and were postfixed with buffered 1% osmium tetroxide and embedded LX 112 (Ladd). Thin sections were cut using an Ultracut E microtome (Reichert-Jung) and poststained with uranyl acetate and lead citrate (Carlsberg system, LKB, Sweden). The electron micrographs were taken with a JEOL 1200EX transmission electron microscope operating at 60 kV.

Immunohistochemistry

The activity of bacteria recognizing receptors in frozen formalin-postfixed histological sections from 8 thrombus aspirates were studied using CD14, clone 7, 1:70 (Novocastra, Leica Biosystems Newcastle Ltd, UK) and CD68, clone KP1, 1:1000 (Nordic Biosite, AIB-30047, Nordic BioSite AB, Sweden), antibodies diluted in Normal Antibody Diluent (BD09-125)

supplied by Immunologic. The diluted antibodies were pipetted onto slides for 40 min and washed in TBS-Tween for 2 x 5 min. Secondary staining was performed with BrightVision+ detection system (DPVB110-HRP) and visualization was done with diaminobenzidine (Bright DAB (BS04-110)) according to the protocol of Bright DAB. Confirmatory staining was prepared with primary antibody replaced with dilute as well as with DAB only to exclude the possibility of any erroneous staining result due to endogenous perioxidase activity or necrotic cells.

Ethical issues

The studies were approved by the Ethics Committees of Satakunta Central Hospital and Turku University Hospital. All patients gave informed consent.

Data analysis

SPSS version 18.0 was used for the statistical analysis (SPSS Inc., Chicago, U.S.A.). The associations between the presence of bacterial DNA (positivity/negativity) in the sample and clinical parameters as well as between the presence of bacterial DNA and dental findings were calculated using Fisher's exact test. To calculate adjusted p-values, binary and multinominal logistic regression analyses were used, where age and sex were used as covariables. Odds ratios and confidence intervals were calculated by CIA software version 1.1[22]. P-values were not corrected for multiple comparisons due to the hypothesis –oriented study approach[23]). A *P*-value less than 0.05 was considered statistically significant.

Results

Patient characteristics

The study included 101 MI patients. Detailed clinical characteristics are presented in Table 1. When we compared the clinical parameters of patients between these two heart centers, no

significant differences ($p<0.05$) were seen, except that dyslipidemia was more common in subjects in center 1 (76.0 % vs. 37.3; $p<0.001$; chi squire). All patients received aspirin and clopidogrel or prasugrel prior to the intervention. Bivalirudin was used in 55.4 % and glycoprotein IIb/IIIa inhibitors in 18.8%.

Bacterial findings in thrombus aspirates

Using real time quantitative PCR, median value for total amount of bacterial DNA in thrombi was 16 -times higher than that found in their peripheral arterial blood samples (median, 25^{th}-75^{th} percentiles 16.2; 2.10-64.39). Figure 2 shows the relative amounts of candidate bacteria measured in their thrombus aspirates compared to that in their arterial blood. The greatest amounts were found in the measurements of *Streptococcus* sp. mainly *Str. mitis* -group and *Str. mitis* & *Str. oralis.*None of the samples contained DNA from *Chlamydia pneumonia.*

Thrombus aspirates were categorized into candidate bacteria positive and negative. Figure 3 shows the frequencies of bacterial DNA positive findings in thrombus aspirates. The most frequently found bacterial DNA was from *Streptococcus sp.* mainly *Str. mitis*-group in 73 out of 101 thrombus aspirates (72.3%). Bacterial DNA from *Aggregatibacter actinomycetemcomitans* was found in 6 aspirates (5.9 %) and *Porphyromonas gingivalis* in 5 aspirates (5.0 %). Positive results from one or more measurements of typical endodontic bacteria [*Streptococcus sp.* (mainly *Str. mitis*-group), *Str. mitis, Str. oralis*, gftP&gftG-streptococcal virulence factor, *Str. anginosus*-group, *Staphylococcus aureus* & *S. epidermidis, Parvimonas micra* or *Prevotella intermedia*] were detected in 78.2% of thrombus aspirates and periodontal pathogens (*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Dialister pneumosintes* or *Treponema denticola)* in 34.7%.

In 16 cases the n-fold difference was between 0 and 2, *i.e.* under the bacterial positivity

limit for thrombi, suggesting possible bacterial DNA in arterial blood but not in thrombus aspirates. Ten arterial blood samples were positive for *Streptococcus* sp. mainly *Str. mitis*-group, one for *Str. mitis& Str. oralis*, one for *Staphylococcus epiderm & S. aureus*, one for *Porphyromonas gingivalis* one for *Prevotella intermedia*, one for *Fusobacterium nucleatum*, one for *Dialister pneumonistes*.

To demonstrate the possible identifiable bacteria structures in the thrombus aspirates, 9 frozen aspirates were also analyzed by transmission electron microscopy. Bacteria-like components were seen in all nine cases and whole bacteria were detected in 3/9 cases (Figure 4).

Using immunohistochemistry, intensive staining of CD14 and CD68 were observed in all 8/8 thrombus aspirates (Figure 5). The presence of bacterial DNA was detected in all those thrombi.

The presence of bacterial DNA and clinical findings

There was a trend of inverse association (p=0.070, Fisher's exact test, Table 2) between the presence of bacterial DNA from *Streptococcus sp.* mainly *Str. mitis*-group and the number of stenotic arteries [narrowing by quantitative coronary angiography of more than 50%]. The percentage of bacterial positivity was lowest (50.0 %) in subjects with three-vessel disease compared to patients with one (74.5%) or two (81.3%) vessel disease. After adjustment with age and sex the association remained (p=0.065, logistic regression). No other associations were found between different bacteria findings and clinical parameters.

Dental pathology and its association with bacterial DNA findings

The panoramic tomographies of the 30 MI patients showed that the most common dental findings were signs of dental treatment; fillings (one or more) in 86.7 %, and previous root canal treatments in 66.7 % and further pathological findings; furcating lesions in 63.3 %, vertical bone

defects in 50.0 %, and periapical abscesses in 46.6 % (Table 3).Of the periapical abscesses 33.3% coincided with previous root canal treatment. There was a significant association between the presence of periapical abscess and *Streptococcus sp.* mainly *Str. mitis*-group (OR 13.2, 95% CI 2.11 – 82.5; p=0.004, Fisher's exact test) in the thrombus aspirates. Of 14 patients with periapical abscess, 12 subjects (85.7 %) were positive for this bacteria group, whereas in subjects without periapical lesions these bacteria were detected in 5 out of 16 cases (31.3%). The relative amount of these bacteria was also higher (median; 25^{th}-75^{th} percentiles 12.7; 2.0-27.55) in patients with periapical abscesses than those without (1.6, 0.16-6.52). Similar results were obtained with endodontic bacteria (OR 7.71, 1.28 - 46.4; p=0.017). After adjustment with age and sex as confounders, both associations remained significant [p=0.003 and p=0.008, respectively logistic regression). There was also a link between periodontal bacteria and periapical abscess (OR 7.00, 1.14 - 43.0; p=0.046, Fisher's exact test) but this did not remain significant after adjustment (p=0.115, logistic regression). No other associations between other bacteria in thrombi samples and dental findings were found.

Discussion

Our results showed that oral bacterial DNA could be detected in coronary thrombus aspirates of MI. Bacterial DNA typical for endodontic infection, mainly oral viridans streptococci, was measured in 78.2% of thrombus aspirates and periodontal pathogens in 34.7%. A recent study by Ohki *et al* reported similar percentages of periodontal bacteria found in the thrombus aspirates of MI patients[18].

The major bacteria found here in thrombus aspirates belonged to the viridans oral streptococci -group. Viridans streptococcus is a pseudotaxonomic non-Linnean term for a group

of human commensals, most commonly found in the oral cavity. Traditionally, six groups have been classified as viridans streptococci: the *Str. mitis* group, *Str. sanguinis* group, *Str. mutans* group, *Str. salivarius* group, *Str. anginosus* group, and *Str. bovis* group [24]. Approximately 98% of oral streptococci belonged to two viridans streptococci groups: *Str. mitis* and *Str. salivarius* groups [25]. Viridans streptococci have traditionally been assumed to be the most important organisms in periapical lesions[26]. Koren *et al.* showed that abundances of *Veillonella sp*. and *Streptococcus sp*. in the oral cavity were linked with their abundance in carotid atherosclerotic plaques[11]. Oral viridans streptococci have several characteristics which may either induce or maintain the atherosclerotic process. These bacteria are able to attach to different surfaces and generate a biofilm enabling other bacteria to infiltrate the tissue[27]. A recent survey showed that oral viridans group streptococci are capable of invading human aortic endothelial cells and triggering the production of inflammatory cytokines and monocyte chemoattractant proteins[28]. Thrombogenic properties of viridans streptococci have also been investigated. Oral streptococci may initiate or contribute to platelet aggregation in coronaries[29]. A surface protein of *Str. gordonii* [30] has been found to bind to the fibrinogen receptor of platelets. These findings suggest that oral viridans streptococci may be more than "innocent bystanders" with an affinity for inflammatory environments.

To evaluate the pathological significance of our bacteria findings, we also immunohistochemically stained thrombi containing not only platelets but also remnants of ruptured plaque with monocyte/ macrophage receptors CD14 and CD68. CD14 functions as a co-molecule for toll-like receptors which detect conserved microbial patterns and endogenous ligands and play a key role in initiating inflammatory responses[31]. It has been shown that *Porphyromonas gingivalis* and oral streptococci induce proinflammatory cytokine release and

accumulation of macrophages through activation of CD14 / TLR2 complex [4, 32,33]. Moreover, immunostaining of CD68 correlates with the extent of inflammation in atherosclerotic lesion[34]. Thus the detection of DNA specific to oral pathogens together with co-stimulation of CD14 and CD68 in thrombus aspirates may suggest that these pathogens disseminate into systemic circulation, migrate to coronary plaques and cause and / or maintain inflammation. To exclude the possibility of any false staining result due to endogenous debris or necrotic cells chromogenic reporter molecule DAB were used. No staining of DAB was seen.

Oral cavity contains almost 1000 different species and there is a predominance of streptococci[27, 35]. The designing primers and probes for different species of streptococci are challenging due to the high sequence similarities. Moreover, unknown sequences may cause unpredictable cross reactivity within measurements. Of streptococci, *Streptococcus sanquinis* and *Str. gordonii*, detected here by measuring streptococcal virulence factors glucosyltransferase *(gtf)* P&G, can be found only in the oral cavity [36]. Thus all the measurements for *gft* virulence factor illustrate DNA originating from an oral source. Streptococci are commonly also found in the upper part of gastrointestinal tract and the respiratory tract [11]. Although the most likely source for bacteria isolated from atherosclerotic samples is the oral cavity, we cannot exclude the possibility of other sources of transient bacteremia such as the gastrointestinal and respiratory tracts.

It is not currently known how these bacteria end up in atherosclerotic lesions. However, bacteremia originating *e.g.* from the oral cavity or from the gut are more common than has been assumed [37]. Viridans streptococci bacteria are the most common bacteria found in peripheral blood samples after tooth brushing and following dental procedures, such as tooth extraction[38]. One of the most common dental operations is root canal treatment. Bacteremia has been

observed in 30 – 54 % of non-surgical root canal treatments[39]. Transient bacteremia is usually subclinical but can be demonstrated by blood cultures taken shortly after dental procedures[40]. In the peripheral blood bacteria are phagocytosed and may subsequently be translocated into the atherosclerotic plaque, or may end up in the plaque directly through the endothelium or via *vasa vasorum* of the coronary artery. Van der Meer *et al.* 2008 [41] showed that T cells specific to microbial species can be found in large numbers in peripheral blood, and that they are abundant in atherosclerotic plaques. During the development of atherosclerotic lesions, which may take many decades, different microorganisms and different populations of T lymphocytes may contribute to the smouldering inflammatory process that characterizes atherosclerotic tissue. Therefore repeated transient bacteremias following dental procedures and / or other bacteria infections during the lifetime may cause accumulation of pathogens in atherosclerotic plaques which may act as booster of the inflammatory process and maintain chronic low-grade inflammation. Rupture of a plaque populated by bacteria with high affinity to platelets could then lead to thrombus formation and subsequently to an acute coronary event.

Not only bacterial DNA but also whole bacteria cells – even living pathogens - have been detected in atherosclerotic samples[42-45]. In our randomly selected thrombus samples, three out of nine cases were found to contain whole (dividing and / or non-dividing) bacteria whereas various bacteria components and DNA were found in all nine cases studied. Electron microscopy was here used to evaluate the presence of whole bacteria and bacterial fragments in the samples. Although it is the most commonly used and the probably best suited for this purpose, evaluation is based only on morphological characteristics. However, we also performed qPCR in the same samples showing the presence of bacterial DNA. Thus, we can conclude that whole bacterial structures can be detected in thrombus aspirates but the detection of bacteria in samples by PCR

does not provide evidence of whole bacteria but correlates with the presence of bacteria residuals in the area.

The severity of the cardiovascular disease measured as narrowed arteries has been shown to be linked to the severity of on-going dental infection measured as alveolar bone loss, pathological periodontal pockets and the number of missing teeth[17]. As well as infection, the burden has been shown to be associated with more severe atherosclerosis [46]. However, this has been questioned. As early as in 1999 Thomas *et al.* showed that the distribution of bacterial DNA did not correlate with the severity or extent of disease[47]. In a recent study by Ohki *et al.* [18] no association was reported between the severity of coronary atherosclerosis and periodontal bacteria. In our study there was an inverse association between the number of stenotic vessels and dental abscess bacteria, suggesting that bacterial infection may be more important in the vulnerable coronary plaque (Karhunen et al 2013, unpublished data). The observed association between the number stenotic arteries and oral viridans streptococci should be interpreted with caution, since the association was a trend and number of stenosed vessels is only a rough estimate of coronary atherosclerosis.

The limitations of the present study include the fact that not all aspirated thrombi could be immunohistochemically studied due to their small size, neither was the composition of our specimens known. Earlier studies have shown components of atherosclerotic plaque in up to 40% of cases. The thrombus itself is erythrocyte-rich (red) in 30-40% and contains only platelets (white) in 60-65% of cases[48, 49]. Panoramic tomographies were not available from all of our patients limiting the statistical power of the analysis, which is reflected in the wide confidence intervals. The results regarding associations should therefore be interpreted with caution. All our patients had acute MI and no control aspiration samples from healthy coronaries could be

obtained. At this point we cannot estimate if the bacteria results were from living bacteria inside of coronary atheroma or whether they were simply fragments from bacterial DNA engulfed by phagocytic cells from circulation without any pathological significance.

Our results suggest an association between dental infection and acute coronary events. Since the primary prevention of coronary events is mainly based on life-style changes, improvements in dental health and dental care could be one major goal of preventive efforts.

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Conflict of Interest Disclosures: None

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Table 1. Characteristics of myocardial infarction (MI) patients

	MI patients (n=101)
Sex (male/female), %	76.2 / 23.8
Age (yrs), mean+sd	63.3 + 12.02
BMI, mean+sd	28.3 + 4.90, n=71
Hypertension, %	44.6
Diabetes, %	17.8
Dyslipidemia, %	56.4
Smoking (0 / 1 / 2)*, %	59.4 / 36.6 / 4.0
CRP [<5 mg/l / >5 mg/l / no data], %	65.3 / 31.7 / 3.0
Total cholesterol (mmol/l), mean+sd	4.8 + 1.24, n=85
HDL (mmol/l), mean+sd	1.3 + 0.49, n=86
LDL (mmol/l), mean+sd	2.8 + 1.07, n=84
Triglycerides (mmol/l), median+IQR	1.4 + 1.10, n=86
STEMI, %	93.1
Number of stenotic arteries†, %	
1	50.5
2	31.7
3	17.8
Culprit lesion, %	
LAD	42.6
LCx	11.9
RCA	39.6
SVG	3.0
DG/IM	3.0
TIMI before, %	
0	83.2
1	3.0
2	5.9
3	7.9
TIMI after, %	
2	6.9
3	93.1
Killip, %	
1	83.2
2	11.9
3	4.0

BMI, body mass index; CRP, C-reactive protein; Dg/Im, diagonal/intermediate; HDL, high density lipoprotein cholesterol; IQR, inter quartile range; LAD, left anterior descending; LCx, left circumflex artery; LDL, low density lipoprotein cholesterol; RCA, right coronary artery; sd, standard deviation; STEMI, ST-elevation myocardial infarction; SVG, saphenous vein graft *0 corresponds non smoker; 1, smoker; 2 ex-smoker
†at least 50% stenosis in RCA, LAD or LCX

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Table 2. Association of clinical parameters with oral bacteria found in thrombus aspirates of patients with ST-elevation MI undergoing primary percutaneous coronary intervention. DG/IM, diagonal/intermediate; LAD, left anterior descending; LCx, left circumflex artery; RCA, right coronary artery; SVG, saphenous vein graft.

	n	Endodontic bacteria* n(%)	p-value§	*Streptococcus* sp. mainly *Str. mitis* – group† n(%)	p-value§	Periodontal bacteria‡ n(%)	p-value§
All	101	79 (78.2)		73 (72.3)		35 (34.6)	
Number of stenotic arteries‖							
1	51	40(78.4)	0.096	38(74.5)	0.070	19 (37.3)	0.623
2	32	28(87.5)		26(81.3)		10 (31.3)	
3	18	11(61.1)		9(50.0)		6 (33.3)	
Culprit lesion							
LAD	43	33(76.7)	0.588	30(69.8)	0.577	15(34.9	0.526
LCx	12	8(66.7)		7(58.3)		4(33.3)	
RCA	40	33(82.5)		31(77.5)		12(30.0)	
DG/IM	3	3(100)		3(100)		2(66.7)	
SVG	3	2(66.7)		2(66.7)		2(66.7)	
TIMI before							
0	84	62(73.8)	0.192	57(67.9)	0.181	25(29.8)	0.171
1	3	3(100)		3(100)		3(100)	
2	6	6(100)		5(83.3)		4(66.7)	
3	8	8(100)		8(100)		3(37.5)	
TIMI after							
2	7	5(71.4)	0.662	4(57.1)	0.393	1(14.3)	0.417
3	94	74(78.7)		69(73.4)		34(36.2)	
Killip							
1	84	64(76.2)	0.571	58(69.0)	0.255	27(32.1)	0.589
2	12	11(91.7)		11(91.7)		5(41.7)	
3	4	3(75.0)		3(75.0)		2(50.0)	

* *Streptococcus sp.* (mainly *Str. mitis*-group), *Str.anginosus*-group, *Staphylococcus aureus, S. epidermidis, Parvimonas micra, Prevotella intermedia*

†*Str. mitis, Str. oralis, Str. gordonii, Str. sanguinis, Str. pneumoniae, Str. salivarius, Str. thermophilus,* uncultured streptococci, *Lactobacillus lactis*

‡*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Dialister pneumosintes* and *Treponema denticola*

§Crude p-values (Fisher's exact test)

‖at least 50% stenosis in RCA, LAD or LCX

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Table 3. Association of dental findings with oral bacteria found in patients with ST-elevation MI undergoing primary percutaneous coronary intervention

	Presence of dental finding	n	Endodontic bacteria* n (%)	OR (95%)	p-value	*Streptococcus* sp. mainly *Str. mitis* – group† n(%)	OR (95%)	p-value	Periodontal bacteria‡ n (%)	OR (95%)	p-value
All§		30	19 (63.3%)			17 (56.7%)			9 (30.0%)		
Fillings	yes	26	17 (65.4%)	1.89	0.611	15 (57.7%)	1.36	0.773	8 (30.8%)	1.60	1.000
	no	4	2 (50.0%)	(0.23-15.74)		2 (50.0%)	(0.17-11.23)		1 (25.0%)	(0.14-18.0)	
Root canal treatments	yes	20	12 (60%)	0.64	0.589	11 (55%)	0.82	1.000	6 (30%)	1.27	1.000
	no	10	7 (70%)	(0.13-3.25)		6 (60%)	(0.17-3.81)		3 (30%)	(0.24-6.82)	
Furcation lesions	yes	19	13 (68.4%)	1.81	0.696	12 (63.2%)	2.06	0.454	8 (42.1%)	7.27	0.100
	no	11	6 (54.5%)	(0.39-8.35)		5 (45.5%)	(0.46-9.30)		1 (9.1%)	(0.77-68.9)	
Vertical pockets	yes	15	10 (66.7%)	1.33	1.000	10 (66.7%)	2.29	0.462	5 (33.3%)	1.56	1.000
	no	15	9 (60.0%)	(0.30-5.92)		7 (46.7%)	(0.52-10.01)		4 (26.7%)	(0.31-7.82)	
Periapical abscess	yes	14	12 (85.7%)	**7.71**	**0.026**	12 (85.7%)	**13.2**	**0.004**	7 (50%)	**7.00**	**0.046**
	no	16	7 (43.8%)	**(1.28-46.4)**		5 (31.3%)	**(2.11-82.5)**		2 (12.5%)	**(1.14-43.0)**	
Caries lesions	yes	13	9 (69.2%)	1.58	0.708	8 (61.5%)	1.42	0.721	4 (30.8%)	1.0	1.000
	no	17	10 (58.8%)	(0.34-7.22)		9 (52.9%)	(0.33-6.17)		5 (29.4%)	(0.2-5.0)	
Root canal treatment with periapical abscess	yes	10	8 (80%)	3.27	0.246	8 (80%)	4.89	0.119	5 (50%)	3.25	0.204
	no	20	11 (55%)	(0.55-19.5)		9 (45%)	(0.82-29.1)		4 (20%)	(0.61-17.3)	
Residual roots	yes	3	2 (66.7%)	1.18	1.000	2 (66.7%)	1.60	1.000	1 (33.3%)	2.13	1.000
	no	27	17 (63.0%)	(0.09-14.69)		15 (55.6%)	(0.13-19.84)		8 (29.6%)	(0.12-38.48)	
Pericoronal lesions	yes	2	2 (100%)	1E+009 (0.00)	0.520	2 (100%)	1E+009 (0.00)	0.492	0 (0%)	0.000 (0.000)	1.000
	no	28	17 (60.7%)			15 (53.6%)			9 (32.1%)		

Values are N (%), p values were calculated with Fisher's exact test, odds ratio (OR)95%CI

* *Streptococcus sp.* (mainly *Str. mitis*-group), *Str.anginosus*-group, *Staphylococcus aureus, S. epidermidis, Prevotella intermedia, Parvimonas micra*

†*Str. mitis, Str. oralis, Str. gordonii, Str. sanguinis, Str. pneumoniae, Str. salivarius, Str. thermophilus,* uncultured streptococci, *Lactobacillus lactis*

‡*Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Dialister pneumosintes, Treponema denticola*

§Dental findings are N of cases

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Figure Legends:

Figure 1. Dental panoramic tomography of the upper and lower jaws. Arrays indicate periapical abscesses.

Figure 2. Relative amounts of bacterial DNA in patients thrombus aspirates. N-fold difference compared to subject's own peripheral arterial blood. Individual values (◆). Bacteria positivity limit (2-fold difference) pointed by arrow. gr, group

Figure3. Frequencies of bacterial DNA positive findings in thrombus aspirates of ST-MI patients using specific primers and probes in RT-qPCR. Blood sample obtained from the arterial sheath before the procedure was used as reference.

Figure 4. Transmission electron micrographs (left) and bacteria qPCR results (right) from the same thrombus aspirates. Arrows indicate bacterial structures. Total amount of bacterial DNA (purple curve) and amount of *Streptococcus* sp. (mainly *Str. mitis* -group *bacteria,* green curve).

Figure 5. Immunohistochemical stainings with CD 14 and CD 68 antibodies of two thrombus aspirates. A-D: Thrombus aspirate comprising tissue fragments from ruptured fibrous cap. E-H: Thrombus aspirate comprising mainly thrombotic material.

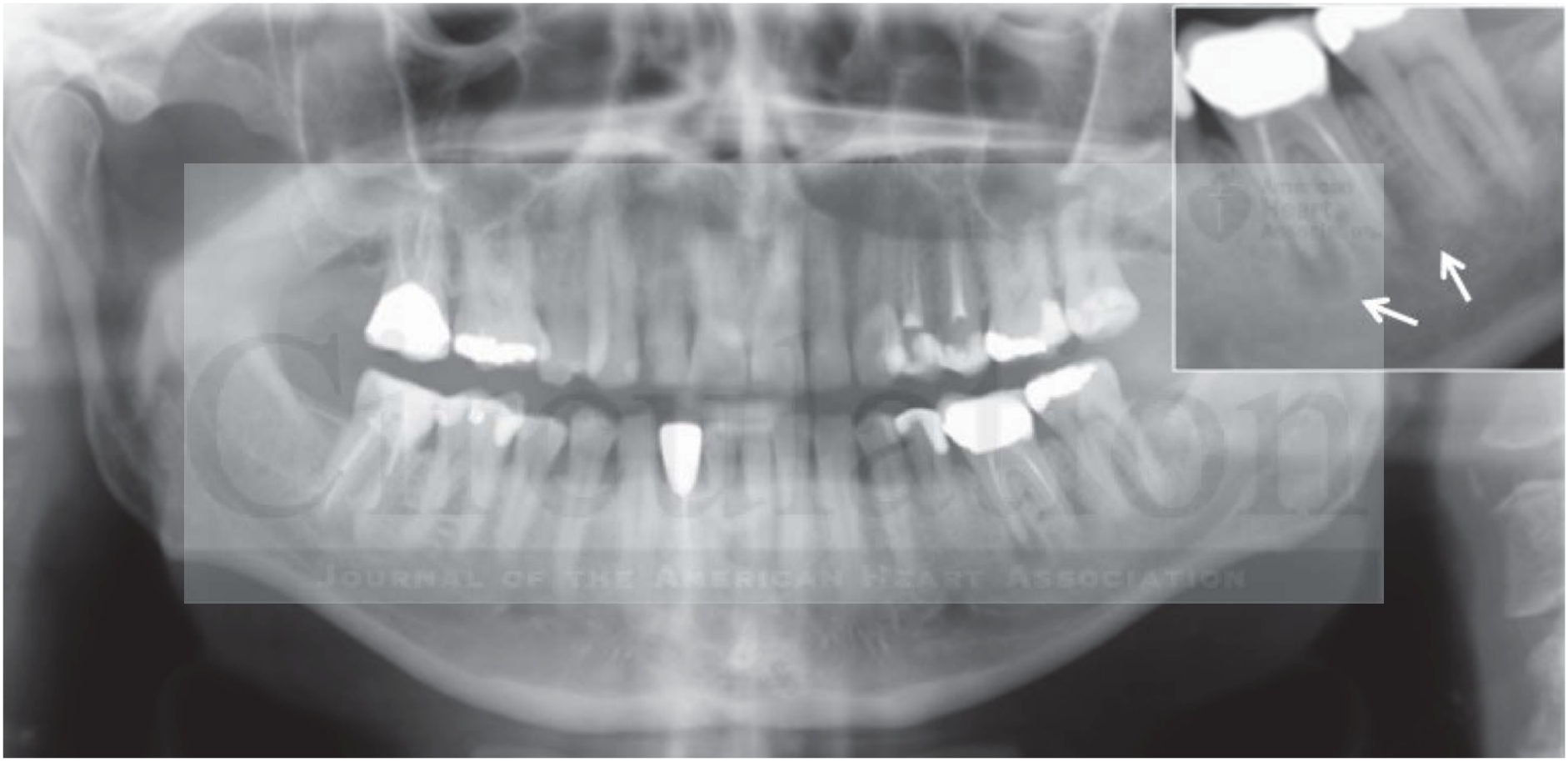


Figure 1

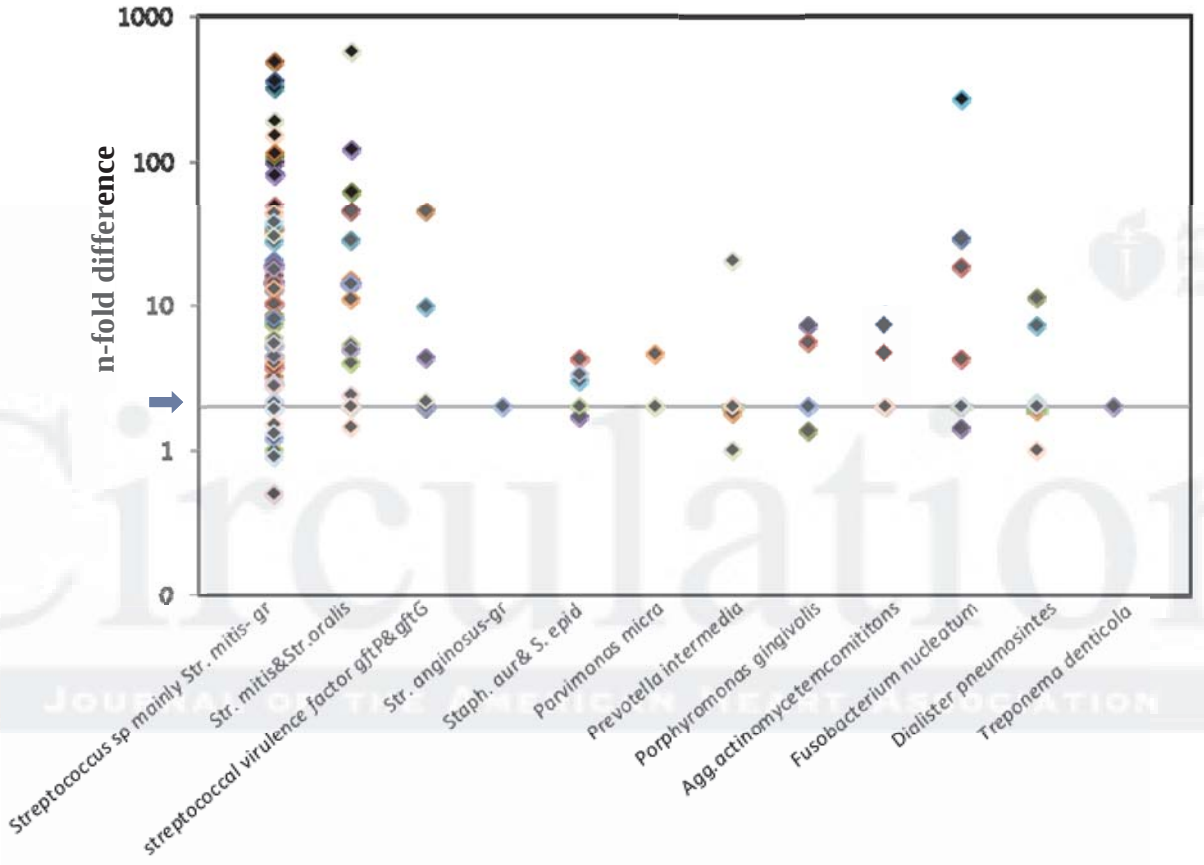
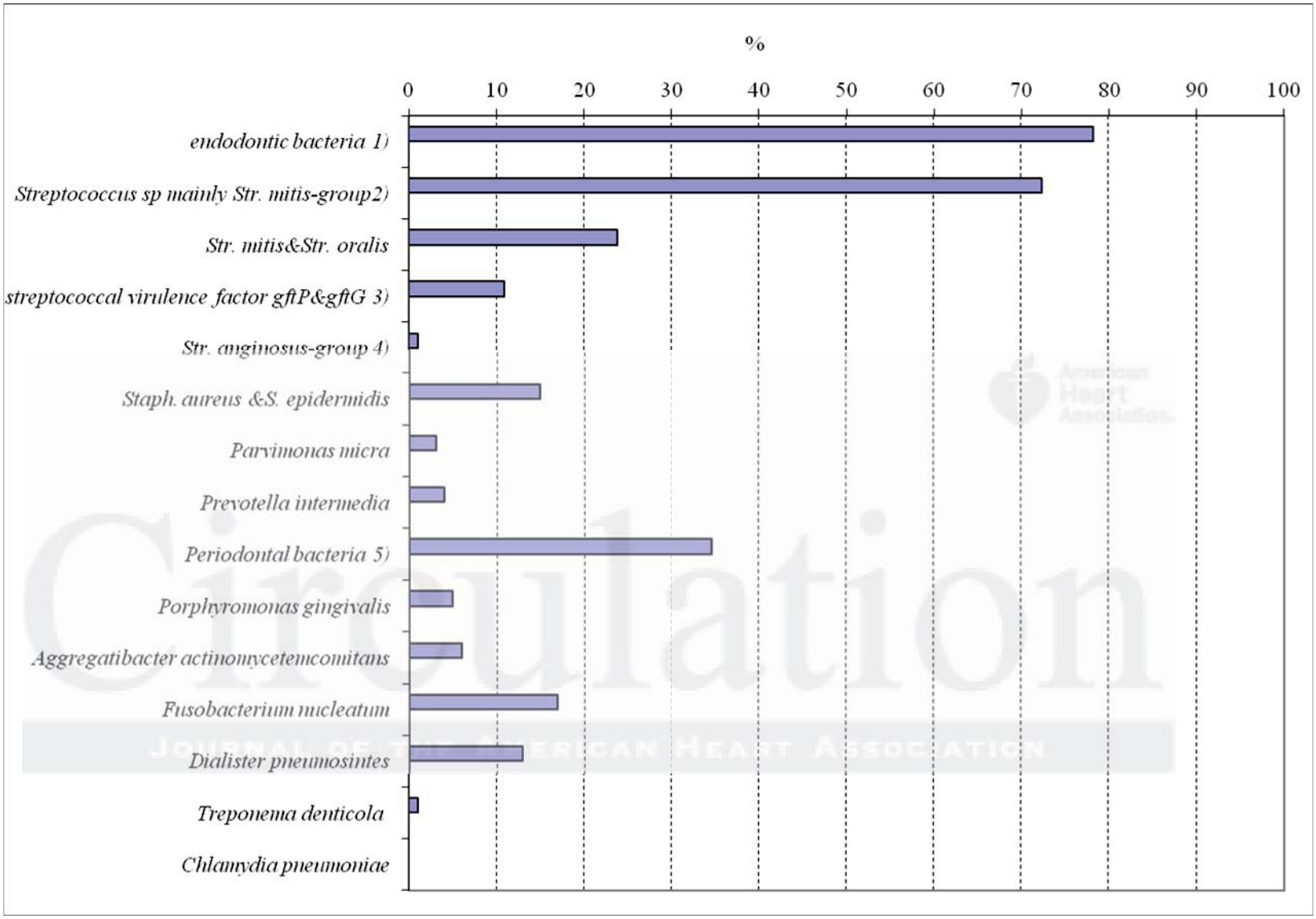


Figure 2



1) positive result from one or more measurements of *Streptococcus sp.* (mainly *Str. mitis*-group), *Str. mitis, Str. oralis*, gftP&gftG-streptococcal virulence factor, *Str. an*
group, *Staphylococcus aureus, S. epidermidis, Parvimonas micra, Prevotella intermedia*
2) recognition of *Str. mitis, Str. oralis, Str. gordonii, Str. sanguinis, Str. pneumoniae, Str. salivarius, Str. thermophilus,* uncultured streptococci, *Lactobacillus lactis*
3) recognition of *Str. sanguinis (gftP) and Str. gordonii (gftG)*
4) recognition of *Str. anginosus, Str. intermedius,* and *Str. constellatus*
5) positive result from one or more measurements of *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Dialister pneumo*
Treponema denticola

Figure 3

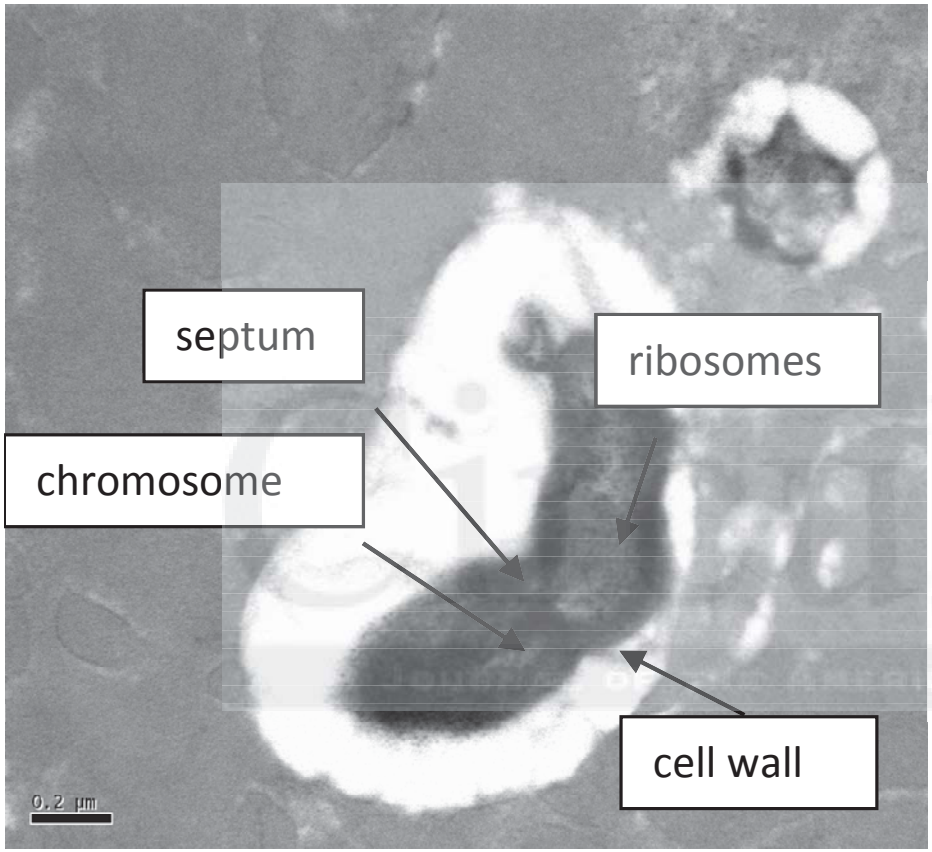


Figure 4

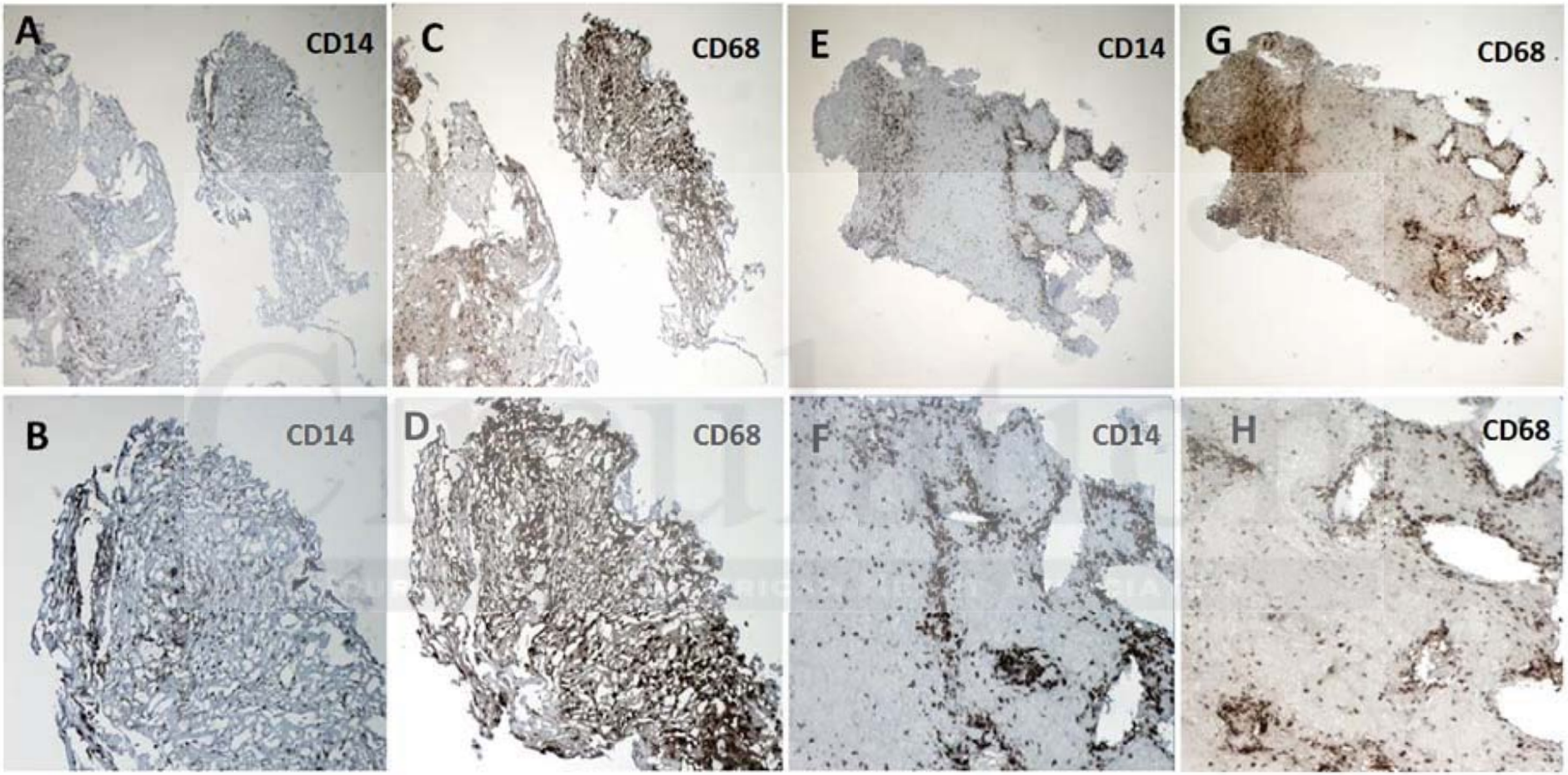


Figure 5

SUPPLEMENTAL MATERIAL

Pessi T, Karhunen V, Karjalainen P et al. Bacterial signatures in thrombus aspirates of patients with myocardial infarction

Supplemental Methods

Supplemental Methods 1. Real time quantitative PCR

Supplemental Tables

Supplemental Table 1. Species-specific primers and Taqman probes for real-time qPCR.

Supplemental References

Supplemental Methods

Supplemental Methods 1. Real time quantitative PCR

For RT-qPCR bacterial DNA was extracted from the samples using a commercially available QIAamp DNA Mini Kit (Qiagen, California, USA) according to the instructions provided. Table 1 presents the oligonucleotide primers and probes used for real time quantitative PCR (qPCR). The primers and probes were designed and confirmed using BLAST with the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov) and/or Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp). Presence and amount of human DNA, total bacterial DNA, candidate bacterial DNA for endodontic bacteria (*Streptococcus sp.* mainly *Str. mitis*-group, *Str. mitis, Str. oralis, Str. sanguinis & Str. gordonii, Streptococcus anginosus* -group, *Staphylococcus aureus, Staphylococcus epidermidis, Parvimonas micra* and *Prevotella intermedia*) and periodontal bacteria (*Porphyromonas gingivalis, Aggregatibacter (néé Actinobacillus) actinomycetemcomitans, Fusobacterium nucleatum, Dialister pneumosintes* and *Treponema denticola),* as well as *Chlamydia pneumoniae* were determined in thrombus aspirates and control arterial blood samples. Specificity and cross reactivity of primers and probes were tested against human DNA, bacteria cultures from clinical samples[1] and reference bacteria from ATCC collection (*Streptococcus mitis* ATCC 49456, *Streptococcus sanguinis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, *Streptococcus anginosus* ATCC 33397, *Prevotella intermedia* ATCC 25611, *Treponema denticola* ATCC 35405, *Dialister pneumosintes* ATCC 33048, *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586, *Parvimonas micra* ATCC 33270, *Aggregatibacter actinomycetemcomitans* ATCC 700685, *Porphyromonas gingivalis* ATCC 33277, *Chlamydophilia (néé Chlamydia) pneumoninae* ATCC 53592; LCG Standards AB, Borås, Sweden). The observed concordance varied between 82.2% and 100%. The cut-off values, *i.e.* detection limits were 40 cycles for all candidate bacteria, except for *Streptococcus* sp. mainly *Str. mitis*-group and *F. nucleatum* whose cut off cycle was 37. The cut-off for universal bacterial measurement was 34

cycles. For each real-time PCR, 5 µl of a mixture containing 1 µl of DNA extract, 1× TaqMan Universal Environmental PCR Master Mix (Applied Biosystems, Foster City, Calif.,USA), 900 nM (each) sense and antisense primer, and 200 nM TaqMan probe were placed in each well of a 384-well plate. Amplification and detection were performed using the ABI PRISM 7900 sequence detection system (Applied Biosystems) with the following cycle profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C for 1 min. PCR was performed twice as duplicates of each sample. In uncertain cases analyses were repeated. The critical threshold cycle (*Ct*) is defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial number of template molecules. Relative amounts of these organisms in specimens were calculated by the comparative *Ct* method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{reference\ sample}$[2]), with a simplification. Briefly, first the differences of Ct values (ΔCt) between candidate bacteria and reference gene for each sample were calculated. Secondly, the comparative Ct (ΔΔCt) for sample (thrombus aspirates) and control samples (blood) was calculated. For the determination of total amounts of bacterial DNA in thrombus, human housekeeping gene, RNaseP (Applied Biosystems) was used as a reference and for amounts of candidate bacterial DNA in thrombus, universal bacteria was used as a reference. The thrombi results were classified into bacteria positive or bacteria negative. Samples were marked positive for candidate bacteria if $2^{-\Delta\Delta Ct} >= 2$ [3, 4] or if there was amplified bacterial DNA in the thrombi but not in the control (blood) sample.

Supplemental Tables

Supplement table 1. Species-specific primers and Taqman probes for real-time qPCR.

Primer and probe	Sequence (5'-3')	reference
***Streptococcus sp.* mainly *Str. mitis*-group** [a]		1
Forward	CCAGCAGCCGCGGTAATA	
Reverse	CCTGCGCTCGCTTTACG	
Probe	ACGCTCGGGACCTACG	
Str. mitis/Str. oralis		5
Forward	GCCATTGAAGCGGTTACTTTG	
Reverse	CATCCGACATTAACGCAAGTTC	
Probe	ATGATTGAGCGTGGAACGGTGGGT	
Streptococcal virulence factor gftG/gtfP [b]		This study
Forward	TTTACCATGGATGAGCTCAAGCAA	
Reverse	TTGGAGAGCATGAGAGCATG	
Probe	ACGCAGTTCAATATCC	
***Streptococcus anginosus* –group** [c]		This study
Forward	CCGTAGTGTTTGTGCTAGGTGAAA	
Reverse	CCAGAGACGTAGCTGTTTCGT	
Probe	CCGTAACGATTTCTCG	
Staphylococcus aureus / S. epidermidis		1
Forward	GCGTTTTTCACGTGGAATATC	
Reverse	AATCCAAAACACAAACAAAGACAAGGT	
Probe	ACGTGCCATATTAATTTAC	
Chlamydophila (néé Chlamydia) pneumoninae		6
Forward	ATCCGCTGCTGCAAACTATACT	
Reverse	TGAACCACTCTGCATCGTGTAA	
Probe	AGGCCGGGTTAGGTCTATCTACGGCAGT	
Treponema denticola		7
Forward	CCGAATGTGCTCATTTACATAAAGGT	
Reverse	GATACCCATCGTTGCCTTGGT	
Probe	ATGGGCCCGCGTCCCATTAGC	
Prevotella intermedia		7
Forward	TCCACCGATGAATCTTTGGTC	
Reverse	ATCCAACCTTCCCTCCACTC	
Probe	CGTCAGATGCCATATGTGGACAACATCG	
Fusobacterium nucleatum		This study
Forward	AGGGTGAACGGCCACAAG	
Reverse	TCTCGGTCCATTGTCCAATATTCC	
Probe	ACACGGCCCTTACTCC	
Aggregatibacter (néé Actinobacillus) actinomycetemcomitans		8
Forward	CAAGTGTGATTAGGTAGTTGGTGG G	
Reverse	CCTTCCTCATCACCGAAAGAA	
Probe	ATCGCTAGCTGGTCTGAGAGGATGGCC	
Dialister pneumosintes		8
Forward	GAGGGGTTTGCGACTGATTA	
Reverse	CCGTCAGAC TTT CGTCCATT	
Probe	CACCAAGCCGACGATCAGTAGCCG	
Porphyromonas gingivalis		8

Forward	TGCAACTTGCCTTACAGAGGG	
Reverse	ACTCGTATCGCCCGTTATTC	
Probe	AGCTGTAAGATAGGCATGCGTCCCATTAGCTA	8
Parvimonas micra		
Forward	AAACGACGATTAATACCACATGAGAC	
Reverse	ACTGCTGCCTCCCGTAGGA	
Probe	TCAAAGATTTATCGGTGTAAGAAGGGCTCGC	8
Universal[d)]		
Forward	TGGAGCATGTGGTTTAATTCGA	
Reverse	TGCGGGACTTAACCCAACA	
Probe	CACGAGCTGACGACA[A/G]CCATGCA	

a) recognition of *Str. mitis*- group (*Str. miti,s Str. oralis, Str. gordonii, Str. sanguinis, Str. pneumonia), Str. salivarius, Str. thermophilus,* uncultured streptococci, *Lactobacillus lactis*

b) recognition of virulence factor for *Str. sanguinis* (gftP) and *Str. gordonii (*gftG)

c) recognition of *Str. anginosus* –group *(Str. anginosus, Str. milleri, Str. constellatus, Str. intermedius)*

d) recognition of total amount of bacterial DNA

Supplemental References

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