UNIVERSITY OF BELGRADE

FACULTY OF BIOLOGY

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PRESENCE OF ORAL PATHOGENIC MICROORGANISMS IN ATHEROMA AND THROMBUS OF PATIENTS WITH ATHEROSCLEROSIS AND MYOCARDIAL INFARCTION

Doctoral Dissertation

BELGRADE, 2020.

UNIVERZITET U BEOGRADU

BIOLOŠKI FAKULTET

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PRISUSTVO ORALNIH PATOGENIH MIKROORGANIZAMA U ATEROMU I TROMBU PACIJENATA SA ATEROSKLEROZOM I INFARKTOM MIOKARDA

Doktorska disertacija

BEOGRAD, 2020.

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Acknowledgments

My deepest gratitude goes to my mentors Professor Jelena Knežević-Vukčević and Professor Jelena Milasin, for their academic guidance, support, and wisdom. I am grateful to my mentors for introducing me to the world of molecular systematic research, and for guiding every step of my way through the Ph.D. I have learned a lot, and your example is an inspiration and something I want to emulate. Thank you for your expert knowledge, invaluable discussions, positivity and continuous support.

I wish to thank also the staff from the Faculty of Biology, Department of Microbiology and the School of Dental Medicine, Department of Human Genetics, of the University of Belgrade for their help and for providing resources for my research.

My warmest thanks goes to all my friends, both living locally and abroad, who have supported, challenged or joined me on this journey.

Yet all of this wouldn't have been possible without the constant support of my family and especially my father and my mother. There are no enough words to say how grateful I am for their tireless support. I also enjoyed an immense support of my wife, sons and daughters. I dedicate this thesis to them.

I am grateful to my Ministry of Education which provided me with the scholarship to support my study.

Presence of oral pathogenic microorganisms in atheroma and thrombus of patients with atherosclerosis and myocardial infarction

Summary

Introduction: Periodontal pockets harbor more than 400 different bacterial species. The aims of this study were to establish new links between periodontitis and atherosclerosis by quantitative and qualitative assessment of periodontal pathogens *Aggregatibacter actinomycetemcomitans* (*Aa*), *Porphyromonas gingivalis* (*Pg*), *Prevotella intermedia* (*Pi*), *Tannerella forsythi* (*Tf*) and *Treponema denticola* (*Td*) in patient's mouth and blood vessel.

Material and Methods: 100 patients with periodontitis and atherosclerosis were included in the study, divided into 6 groups according to the localization of atheromas and into 2 groups in relation to their age (below and over 60). 13 thrombi from patients with myocardial infarction were analyzed for the presence of oral bacteria and CMV. Pathogen presence and their total load were determined by PCR and real-time quantitative PCR, respectively, and statistically analyzed.

Results: 91% of oral samples and 65% of samples of atherosclerotic plaques were positive for at least one of the tested microorganism. Decreasing frequencies of bacteria in oral vs. artery specimens were: *Tf* (76%:53%), *Pi* (71%:31%), *Pg* (60%:38%), *Aa* (39%:14%) and *Td* (21%:6%). and their ratio was as follows: *Tf* 1.4, *Pg* 1.6, *Pi* 2.3, *Aa* 2.8 and *Td* 3.5. Oral bacteria were the most frequent in coronary arteries. There was no statistical difference in the load of microorganisms between younger and older patients, but *Pi* and *Aa* were less frequent in older patients. CMV was more frequent in thrombi than in atheromas.

Conclusion: Oral pathogens were found at high frequency in atheroma plaques and thrombi, with a slightly changed bacterial composition compared to oral biofilm. Patient's age and the distance of a given blood vessel from the mouth influenced microbiological findings.

Keywords: oral pathogenic microorganisms, periodontal disease, atherosclerosis, age, PCR, real-time PCR

Scientific field: Biology

Narrower scientific field: Microbiology

Prisustvo oralnih patogenih mikroorganizama u ateromu i trombu pacijenata sa aterosklerozom i infarktom miokarda

SAŽETAK

Uvod: Parodontalni džepovi sadrže više od 400 različitih bakterijskih vrsta i sa ovom studijom se želela ustanoviti nova veza između parodontopatije i ateroskleroze koristeći kvalitativno i kvantitativno određivanje prisustva parodontopatogena *Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Tannerella forsythi (Tf) i Trepondema denticola (Td)* u usnoj duplji i krvnim sudovima pacijenata.

Materijal i metode: U ispitivanje je bilo uključeno 100 pacijenata sa parodontopatijom i aterosklerozom, podeljenih u 6 grupa u skladu sa lokalizacijom ateroma i u 2 grupe u odnosu na starost pacijenata (ispod i iznad 60 godina). Analizirano je i 13 trombova od pacijenata sa infarktom miokarda na prisustvo oralnih bakterija i CMV. Detekcija patogena i ukupno opterećenje mikroorganizmima određeni su PCR-om (običnim, odnosno kvantitativnim) a dobijeni podaci su statistički obrađeni.

Rezultati: Ispitani mikroorganizmi su bili visoko zastupljeni kako u gingivalnom tako i u aterosklerotskom plaku. Redosled opadajućih učestalosti bakterija u oralnim uzorcima nasuprot ateromima bio je: *Tf* (76%: 53%), *Pi* (71%: 31%), *Pg* (60%: 38%), *Aa* (39%: 14%) i *Td* (21%: 6%), a njihov odnos je bio sledeći: *Tf* 1,4, *Pg* 1,6, *Pi* 2,3, *Aa* 2,8 i *Td* 3,5. Oralni patogeni bili su najčešći u koronarnim arterijama. Nije bilo značajne razlike u ukupnom sadržaju mikroorganizama između mlađih i starijih pacijenata, ali *Pi* i *Aa* su bili češći kod mlađih pacijenata. CMV je bio češći u trombovima nego u ateromima.

Zaključak: Oralni patogeni su bili učestali i u ateromima i u trombovima, sa blago izmenjenim sastavom bakterijske zajednice u odnosu na zajednicu u oralnom biofilmu. Mikrobiološki nalaz zavisio je od starosti pacijenta i udaljenosti krvnog suda od usne duplje.

Ključne reči: oralni patogeni mikroorganizmi, parodontitis, ateroskleroza, starenje, PCR, PCR u realnom vremenu

Naučna oblast: Biologija

Uža naučna oblast: Mikrobiologija

CONTENTS

1. INTRODUCTION

Oral health is continuously classified as one of the most important public health issues globally. Even with substantial improvements of our knowledge related to oral health and cures of oral conditions over the past decades, gingivitis and periodontitis are still among the most prevalent bacterial infections in humans. There has been a considerable rise in the number of people preserving more of their natural teeth, over the past half century; hence, this ever more dentate population is at higher risk of developing periodontal diseases.

The oral cavity is a noteworthy contributor to total burden of infection and inflammation and, consequently to overall health. The mouth harbors more than 700 different bacterial species while periodontal pockets contain more than 400 **(Inaba & Amano 2010**).

Association between oral infections and systemic diseases has been suspected for centuries. The effect of oral health on the rest of the body was proposed by Assyrians as early as the 7th century B.C. **(Malhotra & Parkash 2013)**, and historically, various explanations have been given as to the origin of atherosclerosis, including issues related to oral health. One hundred years ago, "focal infection" hypothesis postulated that caries was etiologically connected to various pathologies in distant human organs **(Batty et al. 2018; Pizzo et al. 2010)** More recently, periodontitis has been linked to the development of atherosclerosis **(Inaba & Amano 2010); (Pihlstrom et al. 2005).** This is still a matter of debate, with the number of various types of studies, addressing the connection between periodontal disease and coronary heart disease, increasing exponentially in the last decades. Several review papers **(Batty et al. 2018; Janket et al. 2003; Lockhart et al. 2012)** have shown that poor oral health is related to increased risk of cardiovascular diseases. The basic explanation for this relationship lies in the evidence that oral infections are linked to increased systemic inflammation **(Batty et al. 2018; Davé & Van Dyke 2008)**, which in turn is considered to be involved in the etiology of cardiovascular disease (**Batty et al. 2018; Davé & Van Dyke 2008).** The high occurrence of coronary heart disease, a leading cause of morbidity burden, if linked with periodontal disease (**Batty et al. 2018; Naghavi et al. 2015)** could markedly be reduced by treating periodontal disease more methodically and aggressively.

1.1. Cardiovascular Disease (CVD)

The leading cause of death worldwide is cardiovascular disease (CVD) and it is accountable for over 17.3 million deaths annually **(Ellis et al. 2017; Mozaffarian et al. 2015; Stringhini et al. 2012; Lopez et al. 2006).** However, quite different trends in CVD mortality are observed in different populations. In high-income countries, CVD mortality has declined progressively over the past three decades **(Stringhini et al. 2012; Levi et al. 2002).** This has been due to positive changes in population lifestyle, i.e. to decreased levels of major cardiovascular risk factors such as tobacco use, hypertension, unhealthy diets and physical inactivity. In parallel there has been an improvement in patients treatment **(Stringhini et al. 2012;** (Unal et al. 2004)**.** While the CVD epidemic is receding in high-income countries, CVD mortality rates tend to be higher in many low and middle-income countries (up to 300-600 CVD deaths per 100,000 population), than in western countries **(Bovet & Paccaud 2012)**. In low and middle-income countries (LMICs), the total CVD burden is predicted to increase due to aging populations and most probably to detrimental lifestyle changes associated with socioeconomic development and urbanization **(Stringhini et al. 2012; Yusuf et al. 2004; Reddy & Yusuf 1998).** Prevention and control of atherosclerosis include adequate medications, healthy diet, physical activity, etc. Establishment of microbial involvement in this disease might additionally lead to new non-invasive intervention strategies.

CVD refers to a group of disorders of the heart and blood vessels and includes high blood pressure, coronary heart disease (CHD), congestive heart failure, stroke, and myocardial infarction. These conditions lie approximately behind 40% of all deaths worldwide, with atherosclerosis being the principal etiologic factor in the majority of cases **(Inaba & Amano 2010; Seymour et al 2009)**.

Atherosclerosis has been defined as a progressive disease that causes focal thickening of large to medium-sized muscular and large elastic arteries. Atherosclerosis is also the most common cause of coronary artery disease (CAD) **(Pucar et al. 2007; Beck et al 2000; Becker et al. 2001).** Factors, such as smoking, diabetes mellitus and periodontitis have been shown to be related to the development of atherosclerosis.

During the development of atherosclerosis plaques build up inside arteries. Arterial plaque consists of cholesterol, lipids, fibrin, calcium, macrophages (foam cells), and other material related to inflammation **(Ellis et al. 2017; Maton, et al. 1993)** (Figure 1). Over time, plaque hardens and narrows arteries leading to heart attack, stroke, or even death. Atherosclerotic plaque formation, particularly in coronary artery and carotid artery diseases have been the subject of extensive studies **(Ellis et al. 2017; Tedgui & Mallat 1999).** Recent molecular studies have established an association of pathogenic bacteria with arterial disease **(Ellis et al. 2017; Lanter et al. 2014; Anand et al. 2014).** The recognised link between poor oral health and myocardial infarction (MI) **(Chukkapalli et al. 2015; Mattila et al. 1989)** prompted a high interest for the relationship between dental infections and CVD. In particular, periodontitis and microorganisms of the dental biofilm have been associated with arterial disease **(Ellis et al. 2017; Soto-Barreras et al. 2012)**. In addition, oral microorganisms can be a serious medical threat for persons with already predisposing heart conditions such as pacemaker implant, heart valve problems, etc **(Chukkapalli et al. 2015; Ito, H.-O. 2006).**

Studies on animal models and *in vitro* experiments determined likely pathogenetic mechanisms underlying the relationship between atherosclerosis and periodontal disease. Namely, bacteria from subgingival plaque along with their endotoxins possibly will disseminate/shed into systemic circulation via damaged gums and have impact on distant organs **(Chukkapalli et al. 2015; Hirschfeld & Kawai 2015).** One of the central issues regarding the relationship between oral health and CHD, are confounding factors **(Batty et al. 2018; Lockhart et al. 2012).** That is, etiological factors associated with both periodontitis and CHD, such as smoking, alcohol intake, diabetes mellitus provide an alternative explanation for the association. In etiologically oriented

randomized controlled trials, investigators typically control for candidate confounding variables in an effort to identify ‘independent' effects **(Batty et al. 2018; Lockhart et al. 2012).** Another, more powerful approach should be to examine the tooth loss - coronary heart disease tie in subjects who do not drink, or smoke, or are free of diabetes, i.e. free of confounding factors.

Normally, the spread of oral pathogenic microorganisms into bloodstream should be limited by the innate host defense system in the oral cavity. In addition, by maintaining intact the gingival epithelium the spread of oral bacteria should be restrained **(Inaba & Amano 2010; Milward et al. 2007).** Despite this protection exerted by the oral epithelium, it has been shown that oral pathogens can invade blood vessels. Interestingly, although DNA from oral bacteria has been found in atherosclerotic plaques, the contribution of these bacteria to plaque formation remained questionable for a long period of time, and some controversies in this regard are still present.

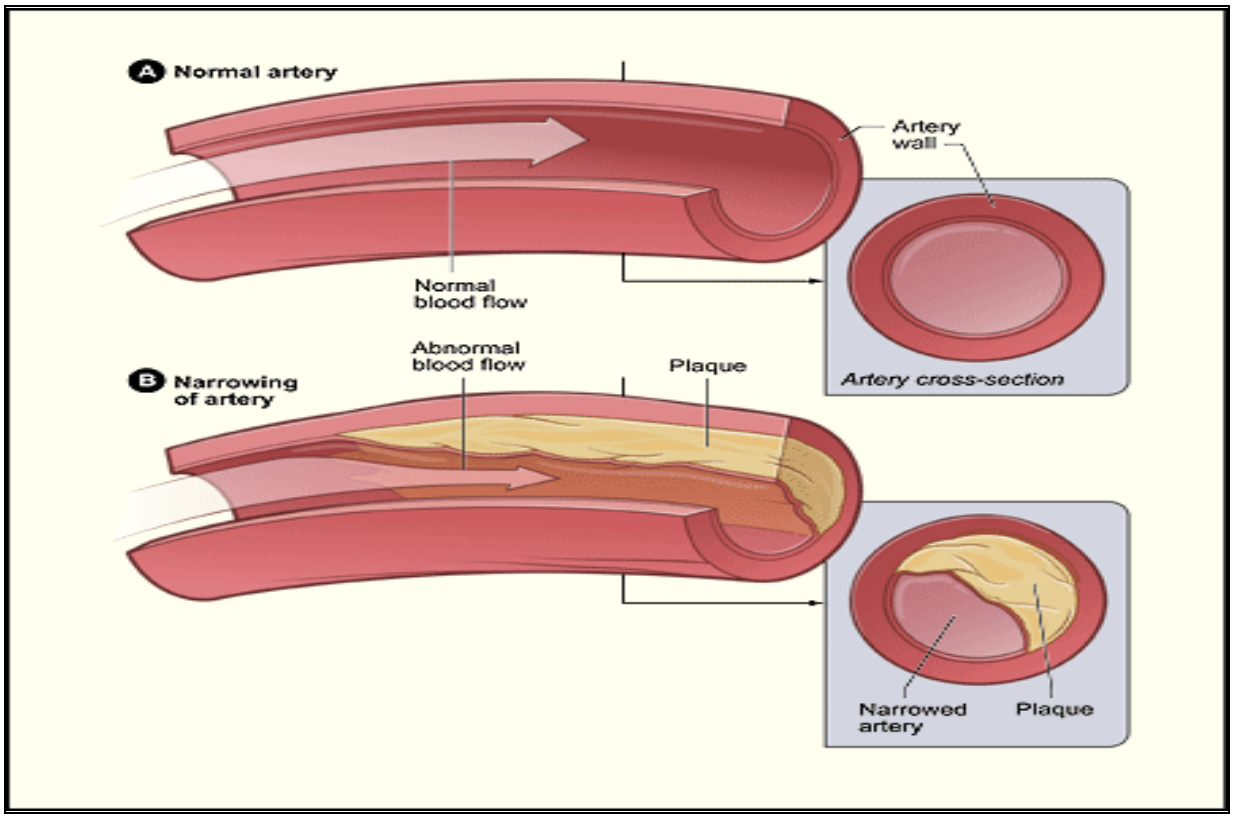


Figure 1. (**A)** Normal artery with normal blood flow; (**B)** Artery with plaque buildup. (https:// www.nhlbi.nih.gov/health/health- topics/topics/atherosclerosis).

1.2. Periodontal disease

Teeth are surrounded by gingiva and the space between the tooth and gingiva is called gingival sulcus and is lined by sulcular epithelium. Within the sulcus, epithelia and crown enamel form specific surfaces inhabited by oral microorganisms. In this specific niche, different bacteria thrive, forming a biofilm termed dental plaque. Primary colonizers in the sulcus are streptococci, for instance *Streptococcus mutans, S. sanguis, S. oralis*, *Neisseria* sp., and others. These microorganisms diminish the local availability of O_2 and hence create a suitable environment for obligate anaerobes that will then invade the gingival sulcus and proliferate there. The first colonizer of the gingival sulcus, beside diminishing O_2, also provide the substrate for bacteria that come later on, which are mostly anaerobic (*Fusobacterium nucleatum, Veillonella sp*. or *actinomycetes* –for instance *Actinomyces odontolyticus and A. naeslundii*, etc.), and form an adequate background for their attachement **(Chukkapalli et al. 2015; Rickard et al. 2003).** It is estimated that oral biofilm bacteria exceed 400 species **(Chukkapalli et al. 2015; Moore & Moore 2000).**

Oral biofilm composition is under the influence of many elements among others O_2 supply, oral hygiene and oral pH, dietary habits and, importantly, the complex relationship between microorganisms. Supragingival and subgingival, microbial contents are significantly different. The subgingival plaque of healthy persons is mostly formed by streptococci, facultatively anaerobic actinomycetes, *and F. nucleatum* **(Chukkapalli et al. 2015; Moore & Moore 2000)** while in the case of gingivitis and periodontitis there is a shift in the microbiome toward predominance of strictly anaerobic species **(Chukkapalli et al. 2015).**

Periodontal diseases, both periodontitis and gingivitis, represent the most frequent dental diseases in human after caries **(Bartova et al. 2014; Milasin et al. 2011; Leong et al. 2014).** The first records on the disease date back to the 9th and 10th centuries A.D. with Arab physicians already relating the condition to soft plaque on teeth **(Bartova et al. 2014)**. Indeed, bacterial plaque, a gluey, colourless film that constantly forms on teeth, is the main cause of periodontal disease **(Inaba & Amano 2010)**.

Gingivitis is the most prevalent and common form of periodontal disease among children and adolescents. It is important to note that it is a reversible pathology. Gingivitis appears as gum swelling, redness, bleeding during tooth-brushing, and may evolve into periodontitis with, in turn, more damage to periodontal tissues and alveolar bone.

Periodontitis is a chronic infection involving teeth supporting tissues in the jaws (both soft and hard tissues). In periodontitis, following the reduction of periodontal tissues, teeth (one or many) become mobile and can be exfoliated **(Leong et al. 2014)**. It is a multifactorial disease and among important elements, besides the presence of pathogenic bacteria, are genetic predisposition and the quality of host immunological reaction to pathogenic microorganisms **(Bartova et al. 2014; Milasin et al. 2011)** Microorganisms of the subgingival plaque, submerged in the extracellular matrix of the sulcular epithelium, establish complex relationships between each other and host cells **(Chukkapalli et al. 2015; Gibbons 1989).** Periodontopathogens have the capacity to enter sulcular epithelium via endocytosis **(Chukkapalli et al. 2015; Meyer & Lippmann 1996)** and this invasion is accompanied by the release of proinflammatory cytokines. Bacterial surface molecules, such as lipopolysaccharides, stimulate the production of cytokines and other mediators of inflammation that will then induce the synthesis of matrix metalloproteinases, enzymes crucial for extracellular matrix remodelling/bone destruction. Ulcerated gingival epithelium releases different chemical mediators such as interleukins, prostaglandin E2, tumor necrosis factor alpha, etc. that will attract neutrophils and macrophages to the lesion site. In physiological conditions, with a healthy immune system, bacterial insult can be maintained under control with no damage to the host tissue. However, if there is an increased bacterial burden or inadequate immune response, the acute

periodontal inflammation becomes chronic. Additional mediators are then synthesized and more cells of the immune system are recruited. The prolonged state of inflammation will induce bone destruction by osteoclasts. The basic processes occuring during the development of periodontal disease, related to both microorganisms and the host are given in Figure 2.

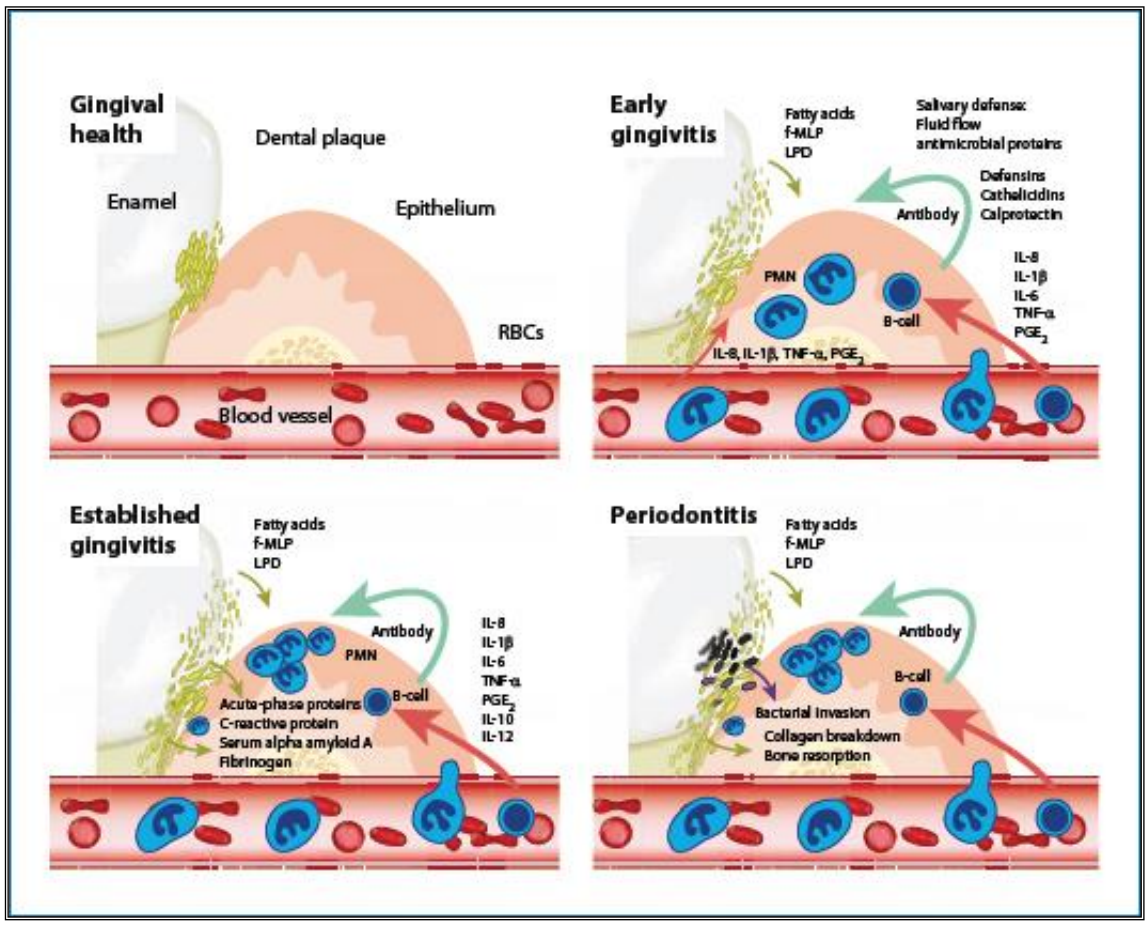


Figure 2. The basic processes occuring during the development of periodontal disease (https://www.dental-nursing.co.uk/features/periodontal-disease-and-systemic-health).

Oral pathogenic microorganisms show a remarkable capacity for survival within the invaded cells. They inactivate antimicrobial factors, disable immune cells, and stimulate processes of host tissue degradation **(Chukkapalli et al. 2015; Amano 2007).**

The deepening of the sulcus, triggered by oral biofilm expansion, local inflammation, and destruction of the gum attachment result in the development of periodontal pockets, site of choice for pathogens accumulation. There, bacteria will exacerbate the inflammation of teeth supporting tissues **(Chukkapalli et al. 2015; Smalley 1994).** In periodontal disease, the fiber attachment is reduced or missing, and the oral biofilm grows apically along the root, initially provoking progressive loss of the alveolar bone around the tooth and lastly of the tooth itself (Figure 3).

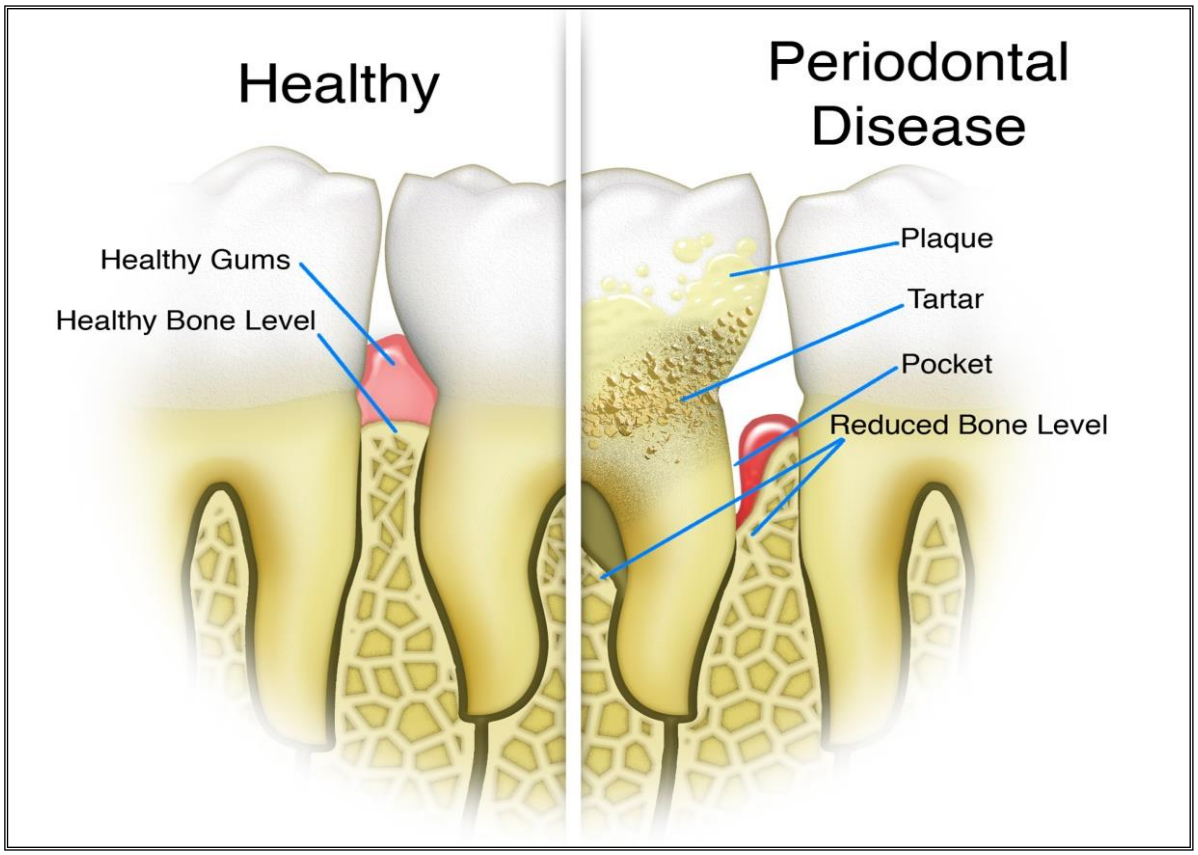


Figure 3. Schematic representation of healthy gum versus gum of a person with periodontal disease (https://toothlessmind.wordpress.com)

Periodontal pockets release crevicular fluid, an exudate that contains bacterial degradation products and metabolites, cell debris, inflammatory molecules, enzymes, etc. **(Chukkapalli et al. 2015; Subrahmanyam & Sangeetha 2003)**. The inflamed periodontal tissue is recurrently (or persistently) bleeding allowing microorganisms to enter the bloodstream **(Pucar et al. 2007).**

1.3. Periodontitis-related microorganisms

Periodontitis is mostly the result of gum colonization by Gram-negative anaerobic bacteria. No single bacterial species has been designated as the primary pathogen; instead, periodontal disease is considered to be a polymicrobial disease. *Porphyromonas gingivalis, Treponema denticola, Aggregatibacter actinomycetemcomitans, Prevotella intermedia* and *Tannerella forsythia* are among those microorganisms strongly implicated in the onset of periodontitis **(Sharma 2010; Socransky et al. 1998).**

Porphyromonas gingivalis (Figure 4) is a Gram-negative, not motile, rod-shaped oral anaerobe belonging to the phylum bacteroidetes. It regarded as one of the major pathogens implicated in the etiology of periodontitis.

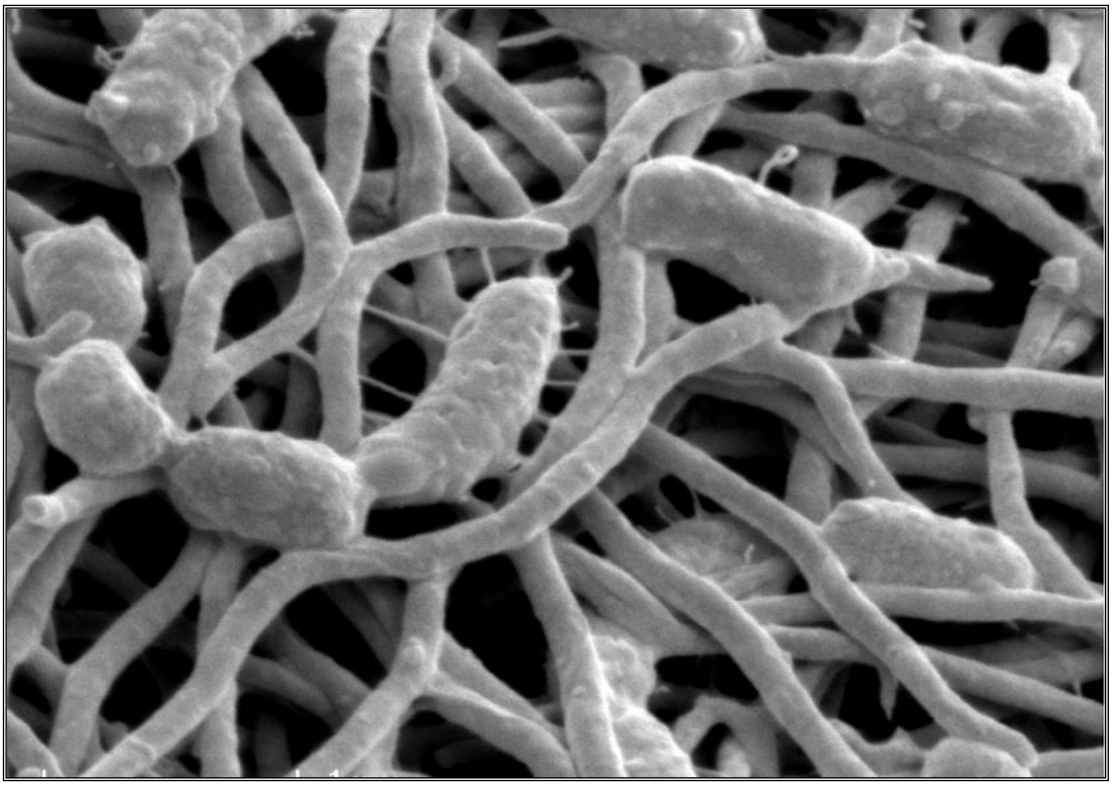


Figure 4. *P. gingivalis* under scanning electron microscope (SEM).
Mary e. Davey, and Margaret j. Duncan j. Bacteriol. 2006; doi:10.1128/jb.01685- 05

Properties and more specifically the pathogenicity factors of *Porphyromonas gingivalis*, which lead to the destruction of tissues surrounding and supporting the teeth, are responsible for the initiation of inflammatory and immune processes **(Mysak et al. 2014; Kinane et al 2008).** *Porphyromonas gingivalis* uses a range of virulence factors that disturb the innate immune system and the host's response to inflammation. It invades periodontal tissues and escapes host defense machinery **(Mysak et al. 2014; Bostanci & Belibasakis 2012)**. *P. gingivalis* activates cellular autophagy and, while suppressing apoptosis, it provides an optimal replicative niche. The severe inflammatory conditions existing in periodontal pockets point to the remarkable ability of this bacterium to respond and adapt to oxidative stress **(Mysak et al. 2014; Henry et al. 2012).** Moreover, it is also clear that *P. gingivalis* has the capacity to avoid host immune response albeit not affecting the inflammatory response, which is beneficial for this and other periodontal

microorganisms **(Mysak et al. 2014; Hajishengallis 2011).** The inflammatory exudate is actually a source of indispensable nutrients. *P. gingivalis* is a keystone pathogen with a disproportionately huge consequence on its environment relative to its quantity, as it is present at relatively low colonization levels **(Andjelkovic et al. 2015; Hajishengallis et al. 2012; Darveau 2009)**. *P. gingivalis* is a quantitatively minor constituent of human periodontitis-associated biofilms, opposing to the findings of early, culture-based, studies **(Kumar et al. 2006; Doungudomdacha et al. 2000)**. The environment of *P. gingivalis* such as host-related factors or other bacteria that are auxiliary microorganisms that help *P. gingivalis* in terms of colonization and metabolic activities, plays an important role in its virulence **(Hajishengallis & Lamont 2012).** *P. gingivalis* virulence proteins such as gingipains, FimA fimbriae, lipid A phosphatase, HtrA protease, etc. have been found to be under the influence of environmental factors (**Xie et al. 1997; Roy et al. 2006).** Moreover, *P. gingivalis* (and other periodontal bacteria) metabolism in affected sites is considerably modified in comparison to healthy gum sites from the same patient (**Jorth et al. 2014)**.

Tannarella forsythia (Figure 5) is an anaerobic Gram-negative bacterium belonging to the *Cytophaga–Bacteroides* family. *T. forsythia* is the only member of the genus *Tannerella*. This microorganism is linked to different forms of periodontal disease (gingivitis, chronic and aggressive periodontitis) **(Sharma 2010; Tanner & Izard, 2006)**.

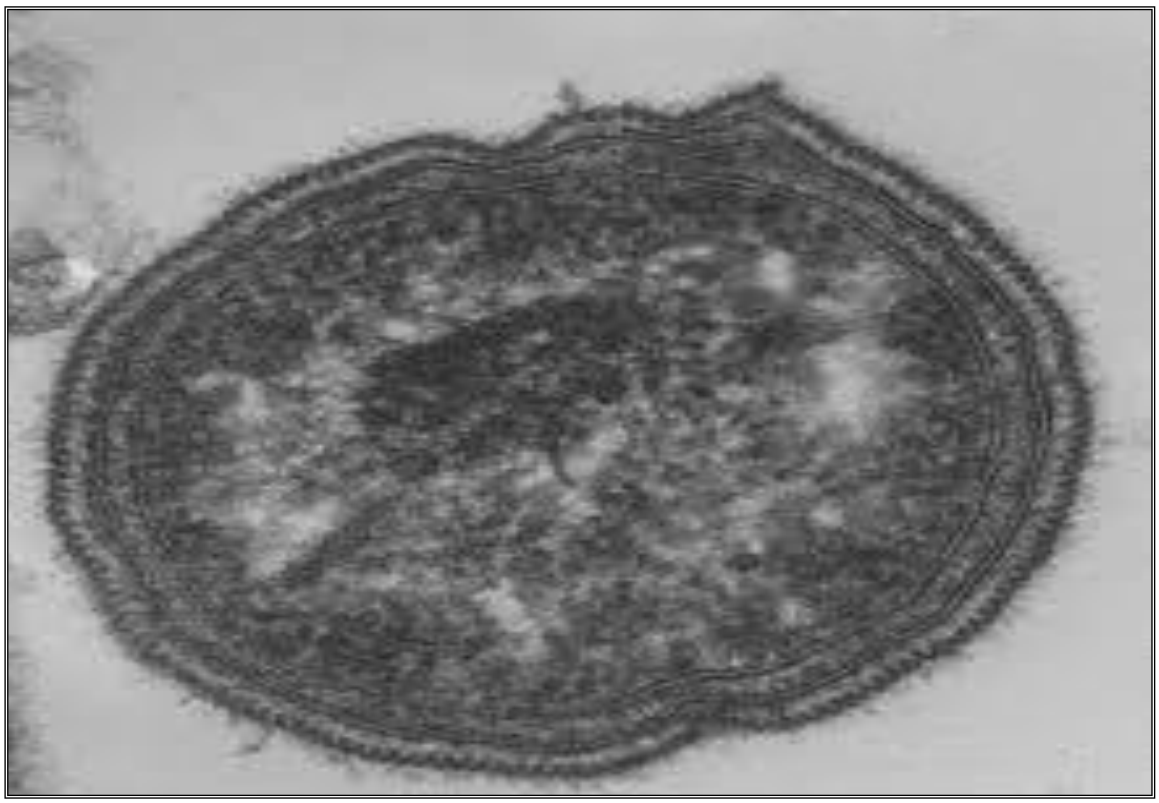
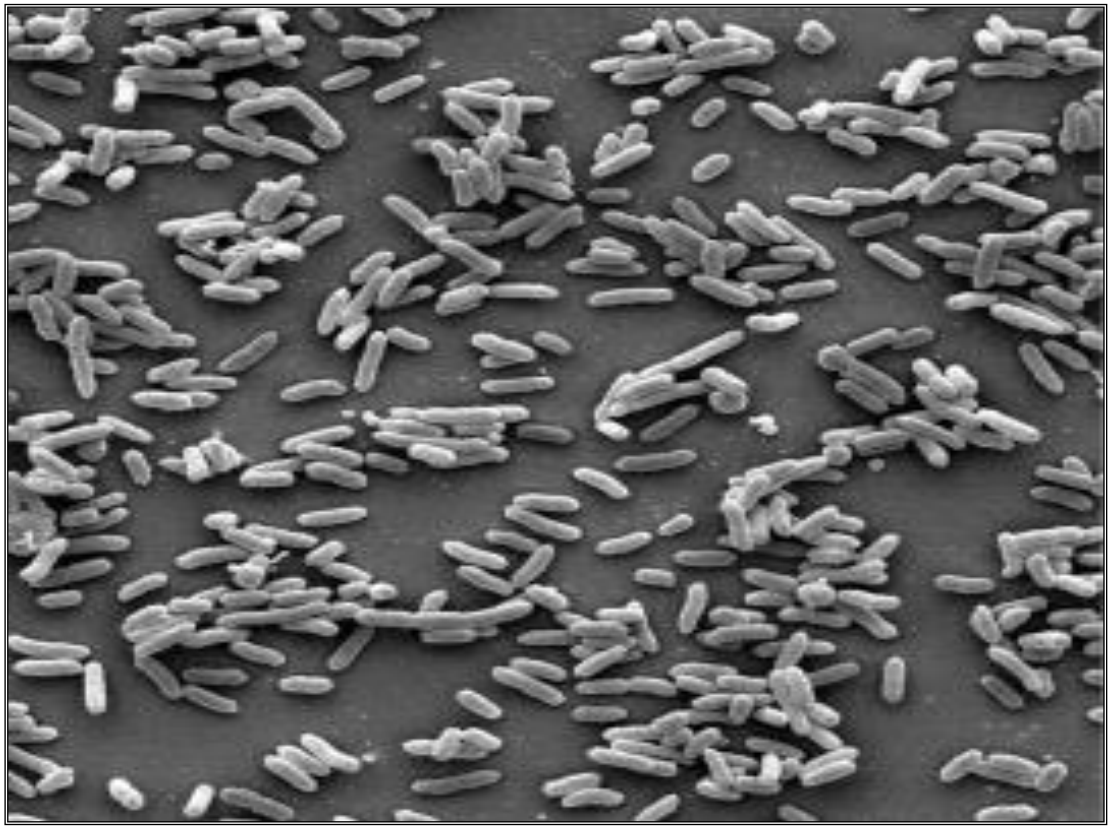


Figure 5. *T. forsythia* seen under transmission electron microscope (TEM). Biology, Medicine. Published in Journal of dental research 2011. DOI:10.1177/0022034510384622.

In spite of considerable evidence connecting *T. forsythia* with the pathogenesis of periodontitis, this microorganism has not been extensively studied mostly due to the very demanding growth requirements in culture and relatively difficult genetic manipulations **(Sharma 2010; Honma et al. 2001)**. Experiments on animal models have clearly shown the virulence of *T. forsythia*; it caused alveolar bone loss in mice and rats **(Sharma 2010; Sharma et al. 2005)**, and this virulence potential was boosted during co-infection with other microorganisms. For instance, abscess development in animal models was increased when *Fusobacterium nucleatum* or *P. gingivalis* were in coinfection with *T. forsythia*. This bacterial partnership was also registered with respect to alveolar bone loss in rats, subsequent to oral infection with the red-complex bacteria (*T. denticola, P. gingivalis* and *T. forsythia)* **(Sharma 2010)**. Probably, *T. forsythia* has a considerable impact on the virulence of concomitant pathogens. Several presumed virulence factors have been recognised in *T. forsythia*, such as trypsin-like and PrtH proteases **(Sharma 2010; Saito et al. 1997; Grenier 1995)**, the sialidases SiaH and NanH **(Brahaml & Moncla 1992; Sharma 2010; Zu et al. 2007)** a leucine-rich repeat cell-surface-associated and secreted protein BspA, **(Sharma 2010; Sharma et al. 1998)**, a-D-glucosidase and N-acetyl-beta-glucosaminidase **(Sharma 2010; Hughes et al. 2003)**, etc. *T. forsythia* surface lipoproteins activate host cells to discharge proinflammatory mediators and induce programmed cell death. It was also established that *T. forsythia* lipoproteins stimulate human gingival fibroblasts and monocytes to produce interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF alpha). Transcription factor nuclear factor-kappa B

(NF- κB**)** activation was shown to be responsible for cytokine synthesis by host cells **(Sharma 2010; Hasebe et al. 2004)**, and it has been proposed that *T. forsythia* lipoproteins are possibly involved in the pathogenesis of periodontal disease by inducing apoptosis.

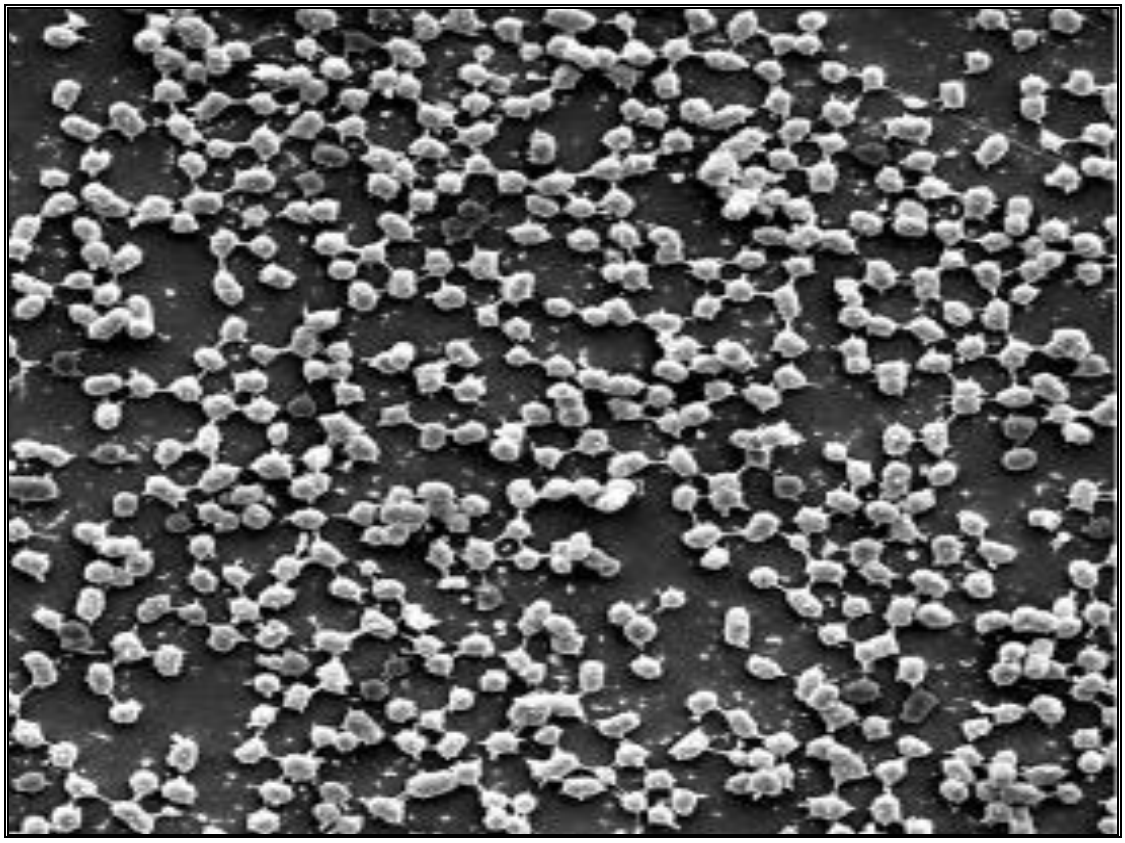
Aggregatibacter actinomycetemcomitans (Figure 6) is a Gram-negative capnophilic, facultatively anaerobic, rod-shaped bacterium belonging to the *Pasteurellaceae* family and HACEK cluster group. The latter stands for *Haemophilus parainfluenzae, H. aphrophilus, A. actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens,* and *Kingella kingae*. This organism was initially named *Actinobacillus actinomycetemcomitans,* but Nørskov-Lauritsen and Kilian (2006) reclassified it as *Aggregatibacter actinomycetemcomitans* (**Nørskov-Lauritsen & Kilian 2006; Hajishengallis et al. 2012).**



Figures 6. SEM micrograph of *A. actinomycetemcomitans.* Subgingival Microbes. (2015). *Atlas of Oral Microbiology, 67–93.* doi:10.1016/b978-0-12-802234-4.00004-5

A. actinomycetemcomitans is one the most important bacteria underlying juvenile, localized aggressive periodontitis. *A. actinomycetemcomitans* strains are divided into 5 serotypes, from **a** to **e**, of different virulence potential **(Gholizadeh et al. 2017)**. The niche of this organism is oral cavity, but it has also been isolated from patients with different infectious conditions, such as septicemia, endocarditis, pneumonia, skin infections, osteitis, etc. *A. actinomycetemcomitans* may be particularly pathogenic because of its ability to invade periodontal tissues. The microorganism's virulence is the consequence of several factors: factors that modulate the promotion of inflammation, factors that induce host tissue destruction, and factors that inhibit host tissue repair. Bacterial virulence products/components include exopolysaccharides, various exotoxins, along with leukotoxin and cytolethal distending poisonous substance, etc.

Prevotella intermedia (Figure 7) belongs to the group of black-pigmented, Gram-negative bacteria. *P. intermedia* is typically related to periodontitis as it has been isolated from deep dental pockets **(Gharbia et al. 1994; Yang et al. 2013).**



Figures 7. SEM micrograph of *P. intermedia.* Subgingival Microbes. (2015). *Atlas of Oral Microbiology, 67–93.* doi:10.1016/b978-0-12-802234-4.00004-5**.**

P. intermedia is regarded as a potent periodontal pathogen responsible for aggressive forms of the disease. However, it has also been found in periodontally healthy subjects. In early studies there was a certain confusion regarding *P. intermedia* and *P. nigrescens*, two closely related species, as they were considered as *P. intermedia* serotypes (serotype 1 and serotypes 2 or 3). Assignment to two separate species was done relatively recently (**Riggio et al. 1998**). *Prevotella* has been very often isolated from polymicrobial infections of the oral cavity, for instance from periapical lesions, periodontal disease related to pregnancy **(Maeda et al. 1998)**, and HIV associated periodontal lesions **(Murray et al. 1989).** *P. intermedia* metabolites are known to initiate and promote oral diseases, both directly and indirectly. *P. intermedia* products and surface components induce strong immune response and consequent periodontal tissue damage. Interactions between fimbria of bacterial and lamellipodia of epithelial cells determine adhesion and invasion capability of *P. intermedia*. Strong bacterial bonding could be explained by stimulation of host cell lamellipodia by damage and inflammation. Due to this interaction, *P. intermedia* serves as a primary colonizer of oral keratinocytes and a fundament for secondary colonizers, such as *P. gingivalis*. This obligate anaerobe is resistant to many antibiotics, but as *P. intermedia* uses iron for its metabolic purposes, it has been shown that oral iron chelators could be a suitable agent for controlling its growth.

Treponema denticola (Figure 8) is a Gram-negative bacterium, belonging to spirochetes. It is an obligate anaerobe, highly proteolytic and motile, residing within the oral cavity and well adapted to survive in this quite specific niche.

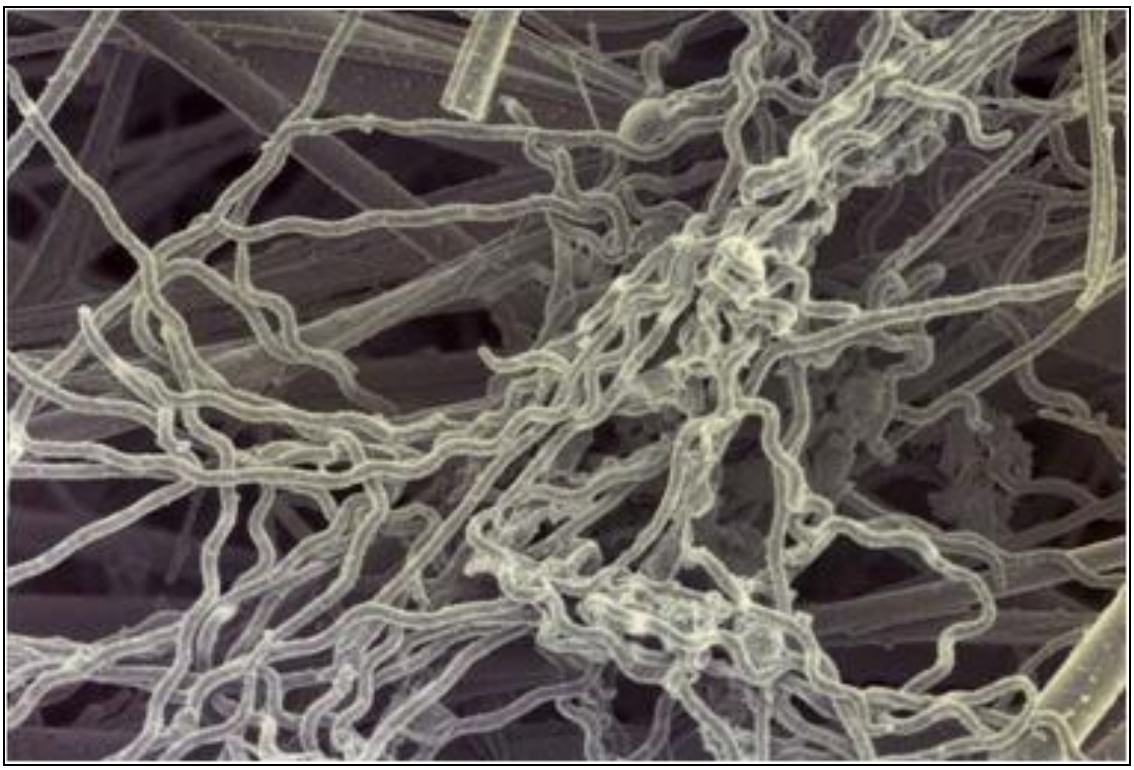


Figure 8. SEM micrograph of *T. denticola.* http://gbpusdchart.com/graph/Treponema+Denticola/

T. denticola is usually isolated from human periodontitis lesions and its involvement within the pathologic process and progression of periodontal disease has been well documented **(Inagaki et al. 2016)**. *Porphyromonas, Tannerella , and Treponema* form the so-called "red complex" and are regularly co-isolated from lesions of chronic periodontal disease **(Socransky et al. 1998)**. So far, many virulence factors of *T. denticola* affecting host cells and tissues have been described. The major outer sheath macromolecule (MSM) induces innate immune responses through TLR2-MyD88 **(Szulc et al. 2015; Dashper et al. 2011)**, affects the migration of neutrophils via taxis, and additionally acts as a pore-forming toxin. Dentilisin, a *Treponema* surface chymotrypsin-like protease is another major virulence factor, allowing host epithelia invasion **(Inagaki et al. 2016)**. Interestingly, *T. denticola* could be detected within the epithelial cells at twenty-four hours following the infection. Epithelia invasion permits not only to evade host immune surveillance, but also to enter into deeper periodontal tissues.

1.4. Periodontitis-related atherogenesis

Subgingival microorganisms in patients with periodontitis provide a major and persistent challenge to the host. A large number of studies have reported a relationship between periodontal disease and vascular disease, in agreement with all the studies implicating chronic infections in the pathogenesis of vascular disease **(Desvarieux et al. 2015; Espinola-klein et al. 2015; Kario et al. 2001).** Periodontitis atherosclerosis syndrome (PAS) was described in the late 1990s and the number of articles devoted to PAS has increased every year, from only four articles in 1998 to almost 4000 at present **(Bartova et al. 2014)**. The majority of initial studies linking the two conditions, relied on indirect measures, such as pocket depth and attachment loss, or dental indices, radiographic findings (bone loss), extent of tooth loss, etc **(Desvarieux et al. 2015; Loesche et al. 1998)** The project named Vascular Disease Epidemiology Study and Oral Infections was specifically designed to study the hypothesis that periodontal infections accelerate the progression of carotid atherosclerosis and increase the incidence of stroke, myocardial infarction, and cardiovascular disease death **(Desvarieux et al. 2015)**. This study has shown a clear link between periodontitis and atherosclerosis.

Porphyromonas gingivalis, *Tannerella forsythia*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans* DNAs have been found in stenotic coronary artery plaque samples, aneurysmal wall and aneurysmal thrombus tissues **(Inaba & Amano 2010; Ishihara et al. 2004; Iwai et al. 2005)**. It is postulated that pathogenic microorganisms from periodontal pockets, along with their products via discontinuous/ulcerated sulcular epithelium, enter into periodontal tissues and further on into circulation. This is followed by systemic inflammation, with increased circulating cytokines and other inflammatory mediators **(Inaba & Amano 2010**; **Seymour et al. 2007; Caesar et al. 2010)**. It can reasonably be expected that this transient bacteremia accompanied with elevated inflammatory markers in patients with periodontitis can influence vascular disease (Figure 9) **(Inaba & Amano 2010; Ebersole et al. 1997)**.

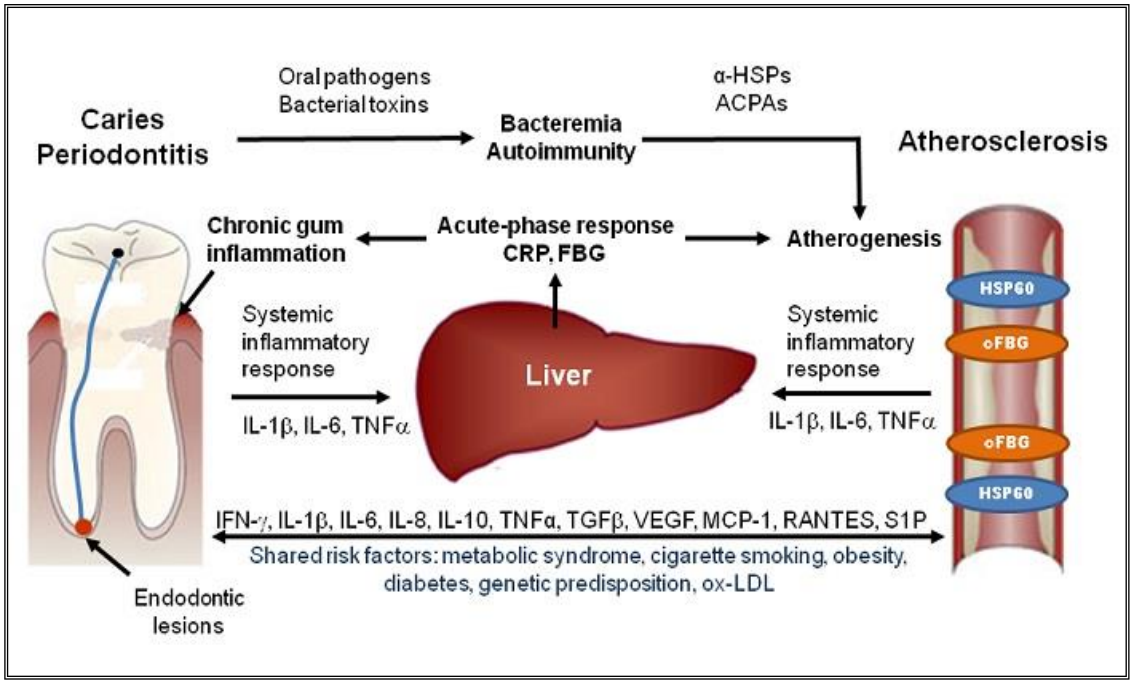


Figure 9. Schematic summary of different effects of oral inflammation on atherosclerosis (source: Aarabi G, Heydecke G, Seedorf U. Roles of Oral Infections in the Pathomechanism of Atherosclerosis. Int J Mol Sci. 2018 Jul 6;19(7))

Different experimental findings have generated different hypotheses of microorganisms' effects on blood vessels. Higashi et al have demonstrated that in patients with periodontitis but without cardiovascular risk factors, endothelial dysfunction was present and was due to systemic inflammation and reduction of nitric oxide availability **(Higashi et al. 2008)**. Other studies have suggested the involvement of endotoxins and antigens produced by periodontal microorganisms in the pathogenesis of endothelial dysfunction **(Bartova et al. 2014; Eng et al. 2015; Maekawa et al. 2010; Bhagat et al. 1996)**. Periodontopathogens have the capacity to up-regulate different chemo-attractant and adhesion molecules of endothelial origin that lead to the attachment of leukocytes onto the surface of endothelial cells **(Schochetman et al. 1988; Bovet & Paccaud 2012)**. *P. gingivalis*, for instance, can induce overexpression of the endothelial monocyte chemo-attractant protein-1 (MCP-1) **(Choi et al. 2005; Hussain et al. 2015)**. In gingival cells, it can also induce a significant increase of expression and release of endothelin-1, a vasoconstrictor associated with CVD **(Ansai et al. 2002; Kebschull et al. 2010)**. Basically, most of the effects exerted by oral pathogenic microorganisms can be grouped into four putative mechanisms of atherogenesis: (1) low-level bacteremia which allows bacteria to enter the circulation and invade the blood vessels' walls; (2) systemic inflammation caused by mediators released from the sites of oral inflammation into the blood stream; (3) autoimmunity to proteins resulting from host immune response to specific components of pathogenic bacteria; (4) pro-atherogenic effects of different toxins produced by oral pathogens. In other words, chronic infections can contribute to atherogenesis either by direct mechansims such as platelet aggregation, invasion of endothelial cells, endothelial injury, etc. or/and by indirect mechanisms such as the production of intracellular adhesion molecules, production of antibodies to lipopolysaccharides, production of cytokines and dysfunction of the immune system **(Buhlin et al. 2009)**.

Although bacterial infections are considered to be the most important, when dealing with the role of oral inflammation in the etiology of atherosclerosis, there are however studies pointing also to the involvement of viral infections in atherogenesis. Herpes simplex virus (HSV) has been proposed in 1970s as a possible causative agent in the development of atherosclerosis in experimental animals **(Fabricant et al. 1978)**. Another member of the herpesviridae family, cytomegalovirus (CMV) has also been implicated. Cytomegalovirus, which causes a lifelong infection, belongs to the subfamily Betaherpesvirinae. CMV has a high prevalence in the human population (55-100%); the marked variation in its prevalence is mainly due to differences in socioeconomic and geographical factors. Primary infection and reinfection of adults occur at higher rates among those in touch with young children within the home, daycare centers and colleges. Children seem to be a very important for CMV shedding, with rates of shedding peaking at 1-2 years **(Amin et al. 2018)**. Human Cytomegalovirus may be implicated in the pathogenesis of periodontal disease as a direct result of viral infection or as a consequence of herpes viral-bacterial co-infection, when virally induced impairment of local host defense favors the overgrowth of pathogenic bacteria. Recent studies have confirmed that destruction of periodontal tissues may be associated with the coexistence of Epstein-Barr and CMV and periodontal bacterial pathogens **(Jakovljevic et al. 2016)**. Thus, oral infections can serve as viral reservoirs and local tissue destruction may lead to virus transfer to blood vessels, along with bacteria.

Many serological and molecular studies have determined the association between CMV and atherosclerosis. More precisely, CMV infection of endothelial cells, leading to endothelial cells damage and metabolic alterations, has been shown to play an important role in the development of atherosclerosis. The development of atherosclerosis related to viral infection, by its consequences in terms of host reaction, resembles to the events caused by bacteria, i.e. it is closely related to inflammatory processes with the production of interleukins (IL-1, Il− 6, Il− 8, etc.), cytokines (TNF-α), endothelial injury, lipid deposition, etc. **(Du et al. 2018)**. In conclusion, some authors claim that viral role is mainly to exacerbate the effects of bacterial infection, but their direct influence on CVD cannot be ruled out.

2. AIMS

The main aim of the present study was to evaluate the changes occurring in the composition of the oral pathogenic microorganisms during aging and their impact on the presence of microorganisms in blood vessels of patients with atherosclerosis.

Specific aims were the following:

1. To assess the epidemiological and sociodemographic characteristics of the patients with atherosclerosis;

2. To determine the frequency of periodontal pathogens *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola and Aggregatibacter actinomycetemcomitans* in the oral cavity and bloods vessels of patients with atherosclerosis;

3. To compare the presence of microorganisms in younger versus older patients with atherosclerosis;

4. To determine the frequency of periodontal pathogens *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola and Aggregatibacter actinomycetemcomitans* in thrombi of a limited number of patients with myocardial infarction;

5. To compare the frequency of oral pathogens and human cytomegalovirus in atherosclerotic vessels and thrombi of a limited number of patients with myocardial infarction.

3. MATERIALS AND METHODS

3.1. Patients

The bulk of the study included a total of 100 patients with moderate to severe periodontal disease and atherosclerosis. Atherosclerosis was clinically diagnosed at the Clinic for Vascular and Endovascular Surgery at the Clinical Center Belgrade, and at the Institute for Cardiovascular Diseases KBC "Dr. Dragiša Mišović" Dedinje in Belgrade.

A positive diagnosis of atherosclerosis was based on:

- Clinical findings,
- Coronary angiography,
- Doppler echosonography

Samples of atherosclerotic plaques obtained after vascular surgery were divided into six groups in relation to the localization of atherosclerotic blood vessel:

- Group I: samples from carotid arteries (30),
- Group II: samples of abdominal aortic aneurysm (10),
- Group III: samples of femoral arteries (10),
- Group IV: samples of iliac arteries (4),
- Group V: samples of coronary arteries (33),
- Group VI: mammary artery samples (13).

In addition to general anamnestic data that included name, sex and age of the patients, the duration of the disease and the possible existence of predisposing factors were registered in the patients' charts. Patients were examined in hospital conditions one day prior to scheduled surgery. Information on education and occupation (social status) and about life style (smoking, alcohol consumption and hygiene habits) were also collected. In the personal history, information about possible systemic diseases (hypertension hematological and endocrine diseases) was also sought. From family history information on the presence of family disorders (CVD, hypertension and diabetes mellitus type 2) was also obtained.

Additionally, a small number of thrombi (13 in total) obtained from patients with myocardial infarction was included in the study and analyzed for the presence of oral pathogens and human CMV.

3.2. Isolation of DNA

For the detection of periodontal pathogens, bacterial DNA was isolated by treating the samples (both subgingival plaques obtained by paper points, and atherosclerotic plaques) with 10% proteinase K (MBI Fermentas, Vilnius, Lithuania) at 56°C for 30 minutes, followed by inactivation of the enzyme by heating the samples at 94°C for 15 minutes. DNA prepared in this way was stored at –20°C prior to PCR analysis.

3.3. Polymerase chain reaction analysis (PCR)

The presence of oral pathogenic microorganisms in subgingival plaques, atheromas and thrombi of patients with atherosclerosis and myocardial infarction was determined by PCR method, using pairs of oligonucleotide primers specific for the given bacterial species.

The standard PCR reaction consisted of the following steps (Figure 10):

Denaturation: extracted DNA is heated, leading to separation of the double strand DNA molecule into two single DNA strands.

Annealing: forward and reverse primers anneal to the complementary site at each of the single-stranded DNA templates. Melting temperature (Tm) of the primer pairs determines the annealing temperature.

Extension or elongation: new complementary DNA strands are synthesized by elongation of the primers thanks to DNA polymerase enzyme.

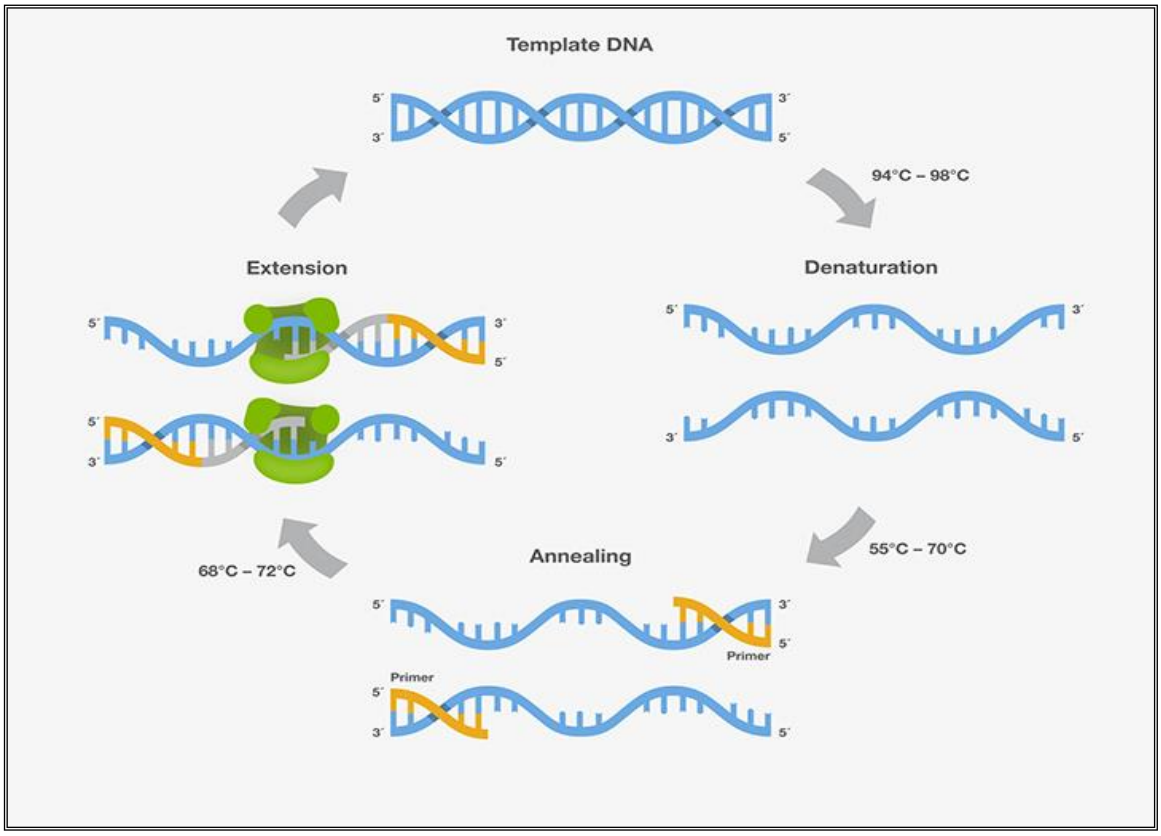


Figure 10. The main steps in PCR: denaturation, annealing, extension

(http://www.sciencemadness.org/talk/viewthread.php?tid=76588)

PCR reaction is carried out with automated thermal cycler with each reaction step at the precise temperature and for a defined duration. Generally, the procedure is repeated 30- 40 times. At the end, the reaction tube contains about 2^{30} molecules of the target PCR product.

From subgingival plaques and atherosclerotic plaques from diseased blood vessels the presence of the following bacteria was investigated: *Porphyromonas gingivalis, Tannarella forsythia, Agreggatibacter actinomycetemcomitans, Prevotella intermedia,* and *Treponema denticola.*

Positive controls were the following: *Porphyromonas gingivalis* (ATCC 33277), *Tannarella forsythia* (ATCC 43037), *Agreggatibacter actinomycetemcomitans* (ATCC 33384), *Prevotella intermedia* (ATCC 33563) and *Treponenema denticola* (ATCC 35405).

PCR was performed in volumes of 25 µl containing 1X PCR buffer (MBI Fermentas), 1.5 mM of $MgCl_2$, 0.2 mM dNTPs, 0.375 µM of each primer, 1 unit of Taq DNA polymerase (MBI Fermentas), and 5 µl of template DNA containing supernatant (Table1). The amplification was performed in a DNA thermal cycler (PeqStar; PeqLab, Erlangen, Germany) programmed at 95°C (3 minutes), followed by a 35 cycle routine of 94°C (45 seconds), annealing temperatures appropriate for each primer pair (Table 2 and Figure 10) (1 minute), and extension at 72°C (1 minute), plus a final extension at 72°C (5 minutes).

Table 1. Final concentrations/volumes of the PCR mixture

Components	**Final concentration**
10 x PCR Buffer	1x
25mM $MgCL_2$	2.5 mM
dNTP	200 µM
Forward primer	0.2 µM
Reverse primer	0.2 µM
Taq polymerase	1 U
DNA	4 µl
Final volume	25 µl

Table 2. The temperature profile for thermal cycle

Initial denaturation	**35 cycles**			**Final extension**
	3 steps			
	denaturation	hybridization	extention	
3 minutes 95°C	1 min 94°C	1 min 55-60°C	1 min 72°C	7 minutes 72°C

Instead of DNA samples, water was used as a negative control for the PCR reactions.

For each microorganism, the DNA obtained from the reference strain was used as the positive control. All primer sequences are listed in Table 3.

Table 3. Primer sequences, specific temperatures of hybridization and length of amplicons for the analyzed bacteria and human CMV

Primer sequence 5'- 3'	**Hybridization (C°)**	**Length of PCR products**
P. gingivalis	55	400 bp
Fwd AGGCAGCTTGCCATACTGCG		
Rv ACTGTTAGCAACTACCGATGT		
A. actinomycetemcomitans	55	500 bp
Fwd GCTAATACCGCGTAGAGTCGG		
Rv ATTTCACACCTCACTTAAAGGT		
T. forsytensis	53	641 bp
Fwd GCGTATGTAACCTGCCCGCA		
Rv TGCTTCAGTGTCAGTTATACCT		
P. intermedia	53	259 bp
Fwd CGTGGACCAAAGATTCATCGGTGGA		
Rv CCGCTTTACTCCCCAACAAA		
T. denticola	53	316 bp
TAATACCGAATGTGCTCATTTACA		
TCAAAGAAGCATTCCCTCTTCTTCTTA		
Human cytomegalovirus		
OUT Fwd CAGTTGACCGTACTGCAC	52	489 bp
OUT Rv GGTATCAAGCAAAAATCT		
In Fwd GAAACGCGCGGCAATCGG	60	301 bp
IN Rv TGGAACTCGAACGTTTGG		

In addition to oral periodontal bacteria, human cytomegalovirus was also analyzed under similar PCR conditions.

3.4. Electrophoresis

Thanks to the speed at which it is performed and its high resolution, polyacrylamide gel electrophoresis (PAGE) is one of the best methods used to separate and visualize nucleic acids. PAGE is based on the copolymerization of acrylamide and bisacrylamide (N-N'-methylene-bisacrylamide). The polymerization is activated by adding TEMED (tetramethylethylenediamine) and APS (ammonium persulphate), the latter containing persulphate free radicals that activate TEMED and the polymerization itself.

In our experiments, products of PCR amplification were mixed with loading buffer, which has a function to increase sample density, color the sample, and allow monitoring of electrophoresis. Electrophoresis was carried out on 8% gel in 1x TBE buffer at a constant voltage of 200V for 60 minutes (Table 4). PCR products were illuminated with ultraviolet light, and according to the size of the amplified DNA sequence (band position on the gel) the product was identified (Figure 11).

Table 4. Components of the polyacrilamide gel used for the visualization of PCR products.

Water	3.9 ml
5 x TBE	1.3 ml
Acrylamide/ Bisacrylamide 30%	1.3 ml
APS 10%	46 µl
TEMED	8.4µl

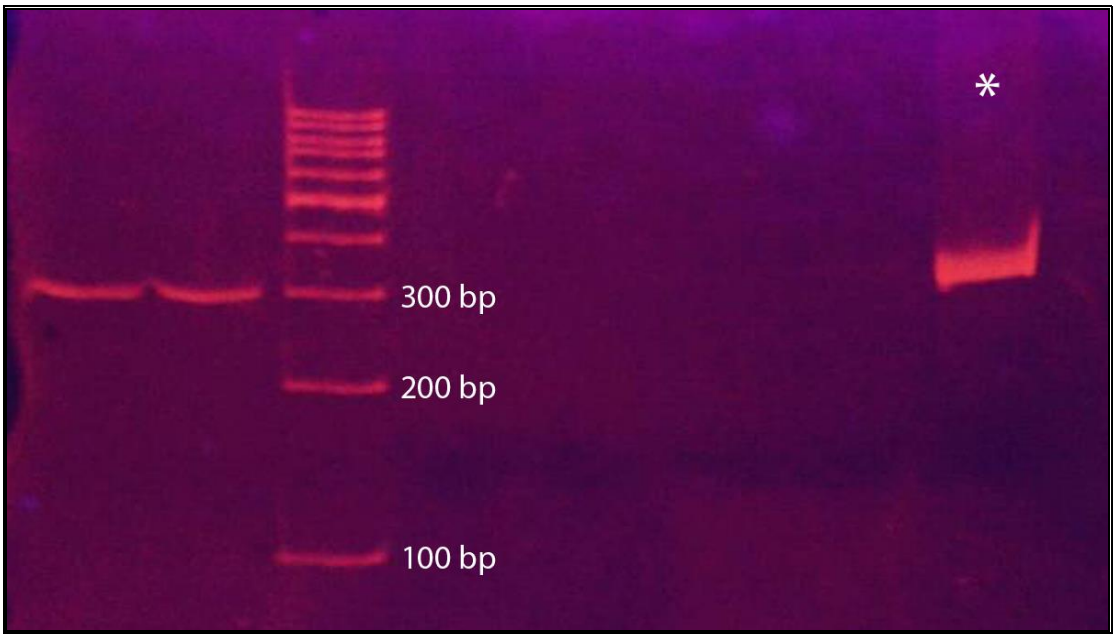


Figure 11. A representative gel showing products of amplification of *T.denticola*; the presence of band (lane 1 and 2) at around 300bp corresponds to the presence of bacteria in the sample; lane marked with * - positive control

3.5. Real-Time Quantitative PCR

For the estimation of total bacterial load in the atherosclerotic plaques and thrombi we used a real-time quantitative polymerase chain reaction method (qPCR). qPCR is a modification of PCR, introduced by Higuchi and coworkers in 1992. It enables exact quantification of specific nucleic acids in a complex mixture even if the starting amount of material is at a very low concentration. Real-time qPCR has become widely used in genotyping analyses, the quantification of viral load in patients, and the estimation of gene copy number in cancer tissue. However, this technology is most commonly used to study gene expression levels by coupling it with the reverse transcription-PCR (RT-PCR) **(Fraga et al. 2014; Ginzinger 2002)**.

The basic principle of qPCR is that as short DNA sequences are copied at each cycle, the amount of DNA in the reaction doubles, resulting in an exponential amplification of the initial target during the early cycles, when the PCR components are largely available compared to the target sequence. But, as product accumulates, the substrates are reduced, resulting in the inhibition of the reaction **(Fraga et al. 2014; Valasek & Repa 2005)**.

The qPCR reaction can be broken into three distinct phases: ***exponential***, ***linear***, and ***plateau***. The first phase of the reaction is the *exponential phase***,** when the reaction is progressing with 100% efficiency and with product doubling at each cycle. Sometimes attaining 100% efficiency is not possible and careful optimization of PCR conditions must be conducted to ensure that reactions are proceeding as efficiently as possible **(Fraga et al. 2014; Guide 2016).** While the amplicon continuously accumulates, the PCR ingredients are consumed, the primer starts competing with amplicon reannealing to itself, and the reaction capability decreases. Over time, the reaction slows down and enters the second phase, the *linear* or *non-exponential phase*. Because of further substrates reduction and product inhibition, the reaction will slow down and enter the *plateau phase***.** Figure 12 represents the three real-time PCR reaction phases and the variations of these phases for different samples **(Fraga et al. 2014).**

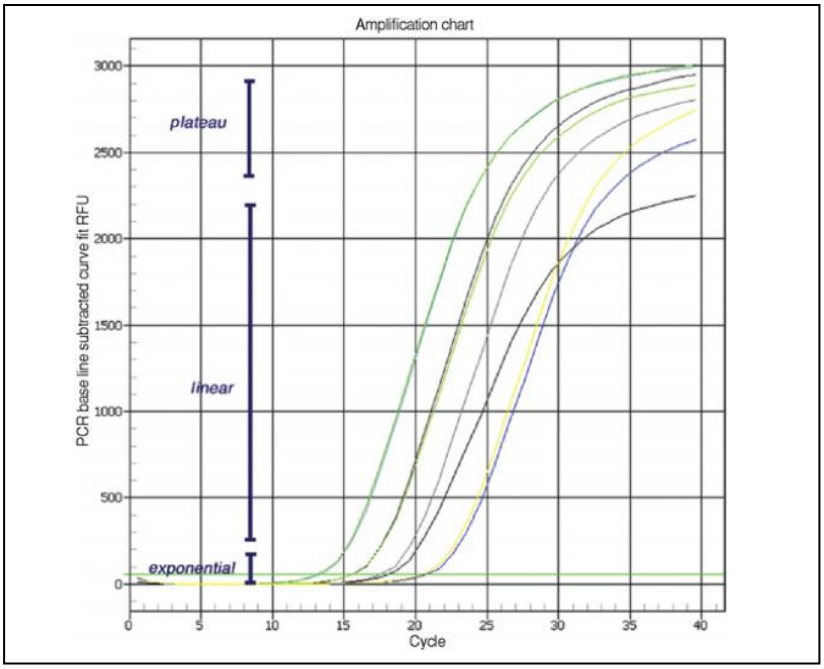


Figure 12. PCR reaction phases, the x axis: cycle number, y axis: amount of DNA

Real-time qPCR measures the amount of amplicon produced during each amplification cycle using fluorescence-based technology and can quantify amplicon production at the exponential phase or the beginning of the linear phase of the PCR reaction, in contrast to measuring the amount of product at the end-point of the reaction. The amplification reaction is monitored by labeling the accumulating product with a fluorescent reporter molecule, such as a double-stranded DNA-binding dye. The typical DNA-binding dyes are SYBR Green I and EvaGreen®. When such dyes are free in solution, they display comparatively low fluorescence, but when bound to double-stranded DNA, their fluorescence increases by over 1000-fold. As the double-stranded DNA increases, the dye binding sites will increase. This dye property provides the mechanism that allows it to be used to track the PCR product accumulation.

As the target DNA is amplified, its increasing concentration in the solution is directly proportional to the fluorescence and can be directly measured by the increase in fluorescence signal; with the qPCR instrument system, the data for each sample during each PCR cycle will be collected. The resulting plots of fluorescence vs. cycle number for all the samples are then set with their background fluorescence at a common starting point (a process known as baseline correction). Then, a threshold level of fluorescence is set above the background but still within the lower part of the linear phase of amplification for all the plots. The cycle number where the amplification plot crosses this threshold, is called the "Ct" or cycle threshold. This Ct value can be directly related to the starting target concentration of the sample. The greater initial DNA template amount in the sample, the Ct value for that sample will be reached earlier **(Fraga et al. 2014; Guide 2016; Arya et al. 2005).** The results are calculated as "relative quantity to the calibrator", where the calibrator sample is assigned an arbitrary quantity of "1" and all the other samples are expressed in terms of their fold difference to this sample.

The earliest approximation method of comparative quantitation (ΔΔCt) is used for this purpose. It utilizes the equation **(Guide 2016; Logan et al. 2009):**

Relative quantity to the calibrator = $2^{-(\Delta\Delta Ct)}$

Where ΔΔCt = (CtGOI – Ctnorm) unknown – (CtGOI – Ctnorm) calibrator

GOI refer to the gene of interest (tested gene), norm refers to the reference gene (or normalizer)

The ΔCt values calculation are illustrated in Figure 13 **(Guide 2016)**.

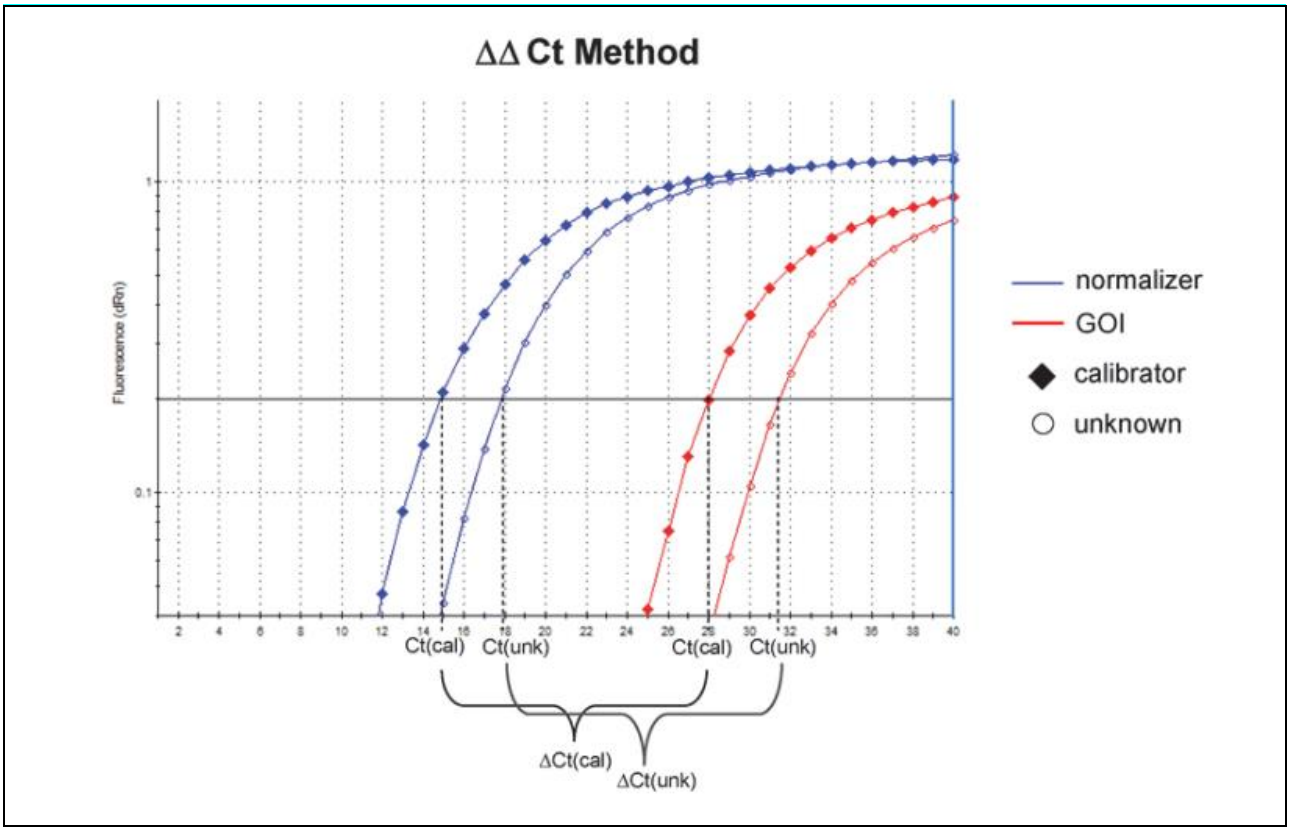


Figure 13. The calculation of ΔCt using ΔΔCt method; ΔCt is determined by calculating the difference between the Ct of the normalizer and Ct of the GOI for each sample (https://pdfs.semanticscholar.org/4a6e/f96ee80979ad3c7799ced161dba37be6c47f.pdf)

In our real-time PCR experiments, reaction mixture (25µl) contained 5 µl of bacterial DNA sample, 12.5 µl of Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania), 1 µl of EuF and EuR primers at the final concentration of 0.2 µM and 5.5 µl of water. The universal primers EuF 5'TCCTACGGGAGCACAGT3' and EuR 5'GGACTACCAGGGTATCTAATCCTGTT3' **(Nadkarni et al. 2002**) (Thermo Fisher Scientific, Vilnius, Lithuania) target highly conserved 16S rDNA region.

Real-time PCR analyses were performed on Line Gene-K Fluorescence Real-time PCR Detection System (BIOER Technology, Shanghai, China) under following conditions: initial denaturation at 94 °C for 10 minutes; 40 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and elongation at 72 °C for 1.5 minutes; and terminal elongation at 72 °C for 5 minutes. qPCR experiments for all samples were performed in duplicate.

A series of ten-fold diluted reference DNA samples (*Prevotella melaninogenica* strain ATCC 25845: 32.4 fg – 32.4 ng) was included in each run in order to generate standard curve. The total bacterial gene copy number was determined by interpolation on the standard curve, as previously described **(Brajović et al. 2016)**.

3.6. Statistical analyses

The methods of descriptive statistics, such as measures of central tendencies, were used to describe the results of the study. The Chi square test was carried out to compare basic epidemiological and clinical characteristics, as well as the prevalence of periopathogenic bacteria between examined groups. A p-value lower than 0.05 was considered statistically significant. All statistical analyses were performed on Statistical Package for the Social Sciences software, version 22.0 (IBM SPSS Inc., Chicago, IL, USA).

4. RESULTS

4.1. Sociodemographic characteristics of patients

4.1.1. Age, gender and lifestyle

The study covered 100 subjects with moderate to severe periodontal disease and atherosclerosis: 69 male (69%) and 31 female (31%). None of the subjects had been treated with antibiotics in the last 3 months nor had received periodontal treatment in the previous 6 months.

The average age of the subjects was 59.2 years. The youngest participant was 28 years old and the oldest 94 years old (Table 5).

Table 5. Age and sex of participants

Sex	Number	$\bar{x}$	SD	Min	Max
Male	69	59.0	12.7	28	94
Female	31	60.0	13.2	32	83

Patients were divided into six groups in relation to the localization of atherosclerosis and into two groups in relation to their age; 28-60 (in total 52, out of which 8 patients were in the group 28-40 years, 17 in the group 41-50 years and 27 in the group 51-60 years) and 61-94 (48 in total, out of which 32 patients were in the group 61-70, 13 in the group 71-80 years, 2 in the group 81-90 years and 1 in the group 91-94 years). The distribution of patients' age in relation to the blood vessel that was atherosclerotic is given in Table 6, and no statistically significant difference could be established among them.

Table 6. Atherosclerotic blood vessels in relation to age

Blood vessel	N^o	$\bar{x}$	SD	Med	Min	Max
Carotid artery	30	61.8	12.1	63.0	28	94
Abdominal artery	10	61.0	7.0	61.5	50	70
Femoral artery	10	55.4	13.4	60.0	32	72
Iliac artery	4	68.0	17.5	69.5	47	86
Coronary artery	33	56.6	13.3	55.0	32	83
Mammary artery	13	57.8	14.8	54.5	42	77

N^o- number, $\bar{x}$ - average, SD - standard deviation, Med - median, Min - minimum value, Max - maximum value

Samples of subgingival plaques and atherosclerotic plaques removed from the diseased blood vessels were examined for the presence of the following bacteria: *Porphyromonas gingivalis*, *Tannarella forsythia*, *Agreggatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Treponema denticola*.

As much as 91% of oral samples were positive for at least one of the tested microorganisms, while in the group of atherosclerotic plaque samples 69% were positive. In subjects with intra-orally present periopathogenic bacteria the average age was 58.8 years, and in subjects with both intra- and extra-orally isolated microorganisms the average age was 59.6 years ($p>0.05$). The average age of subjects with non-isolated microorganisms was 57.3 years.

The majority of participants had secondary education (63%); highly educated participants had a frequency of 24%, while the lowest percentage of participants was with elementary education (13%). No correlation could be established between education and microorganism presence.

More than half of the participants (58%) were smokers, and their distribution in relation to the blood vessels affected by atherosclerosis is given in Table 7.

Table 7. Smoking status in relation to blood vessels affected by atherosclerosis

Blood vessel	**Smoking**	
	Yes (%)	**No (%)**
Carotid artery	76.0	24.0
Abdominal artery	60.0	40.0
Femoral artery	60.0	40.0
Iliac artery	50.0	50.0
Coronary artery	35.0	65.0
Mammary artery	75.0	25.0
Total	58.0	42.0

The incidence of smokers in patients with intra-oral and extra-oral microorganisms was 56.5%, while the incidence rate was slightly lower in patients with only intra-oral microorganisms (55%). Interestingly, the highest incidence of smokers was in the group of patients without detected microorganisms (75%). The frequency of smokers did not differ statistically from non-smokers regarding the presence of microorganisms in the blood vessels ($p = 0.583$).

Alcohol consumption was less represented; only 28% of patients reported that they consumed alcohol and their distribution in relation to the blood vessels affected by atherosclerosis is summarized in Table 8.

Table 8. Alcohol consumption among patients with different blood vessels affected by atherosclerosis

Blood vessel	**Alcohol consumption**	
	Yes (%)	**No (%)**
Carotid artery	38.0	62.0
Abdominal artery	40.0	60.0
Femoral artery	10.0	90.0
Iliac artery	25.0	75.0
Coronary artery	17.0	83.0
Mammary artery	38.0	62.0
Total	28.0	72.0

The frequency of alcohol consumption in subjects without microorganisms was 25%, with intra-oral bacteria 30%, and with both intra-oral and extra-oral bacteria 27%. The frequency of alcohol consumption did not differ statistically between the groups ($p = 0.959$).

Just under half of the participants had a good level of oral hygiene (47%), while 53% of the subjects had poor oral hygiene. The distribution of patients with good or poor oral hygiene depending on the blood vessel affected by atherosclerosis is given in Table 9.

Table 9. Oral hygiene among patients with different blood vessels affected by atherosclerosis

Blood vessel	**Level of hygiene**	
	Good (%)	**Poor (%)**
Carotid artery	31.0	69.0
Abdominal artery	40.0	60.0
Femoral artery	50.0	50.0
Iliac artery	50.0	50.0
Coronary artery	59.0	41.0
Mammary artery	62.0	38.0
Total	47.0	53.0

The frequency of good oral hygiene in subjects without microorganisms was 50%, with intra-oral microorganisms 55%, and with intra-oral and extra-oral bacteria 44%. The frequency of good oral hygiene did not differ statistically between the groups.

4.1.2. Personal history of diseases

Sixty six percent of the participants had been diagnozed with hypertension, and their distribution in relation to the blood vessels affected by atherosclerosisis is given in Table 10. Although a vast variation in the frequency of hypertension was evident (from 100% in the group with iliac artery atherosclerosis to only 30% in the group of femoral artery atherosclerosis), statistically significant difference between the groups could not be confirmed.

Table 10. Hypertension and type of blood vessel affected by atherosclerosis

Blood vessel	**Hypertension**	
	Yes (%)	No (%)
Carotid artery	72.0	28.0
Abdominal artery	70.0	30.0
Femoral artery	30.0	70.0
Iliac artery	100.0	0.0
Coronary artery	62.0	38.0
Mammary artery	75.0	25.0
Total	66.0	34.0

Hypertension in the personal history of the subjects was the least represented in the group of patients without microorganisms. The frequency of hypertension in these subjects was 37%, with intra-oral microorganisms only 65%, and with both intra-oral and extra-oral bacteria it was 69%. The frequency of hypertension did not differ statistically between the groups (P=0.205).

The incidence of endocrine diseases in personal history was the lowest in the group of patients without microorganisms, 25%, followed by 30% in subjects with intraoral findings of microorganisms and, finally in subjects with intraoral and extraoral bacteria, it was slightly higher (32%.). Their distribution according to affected blood vessels is given in Table 11.

Table 11. Endocrinological diseases in personal history and type of blood vessel affected by atherosclerosis

Blood vessel	**Endocrinological diseases**	
	Yes (%)	No (%)
Carotid artery	34.0	66.0
Abdominal artery	40.0	30.0
Femoral artery	25.0	75.0
Iliac artery	25.0	75.0
Coronary artery	21.0	79.0
Mammary artery	50.0	50.0
Total	33.0	67.0

Haematological and immunological diseases in personal history were present only in the group of subjects with intraoral and extraoral findings of microorganisms, in 10% of patients.

Table 12. Hematological and immunological diseases in personal history and type of blood vessel affected by atherosclerosis

Blood vessel	**Hematological and immunological diseases**	
	Yes (%)	No (%)
Carotid artery	30.0	70.0
Abdominal artery	20.0	80.0
Femoral artery	0.0	100.0
Iliac artery	0.0	100.0
Coronary artery	3.0	97.0
Mammary artery	0.0	100.0
Total	6.0	94.0

4.1.3. Familial history of diseases

Data about medical history in the family of the patients are given in Tables 13 (cardiovascular disease) and 14 (diabetes mellitus).

Sixty seven and forty percent of patients had a familly history of cardiovascular diseases and diabetes mellitus, respectively. The family history of cardiovascular disease varied from 50% in the group with iliac artery atherosclerosis, to as high as 83% in the group with carotid artery atherosclerosis. Diabetes mellitus was less represented in the family history: from 24% in patients with coronary artery atherosclerosis, to 55% in patients with carotid artery atherosclerosis. However, there were no statistical differences in the incidences of positive family history in relation to the blood vessels affected by atherosclerosisis.

Table 13. Presence of cardiovascular diseases in the families of patients with different blood vessels affected by atherosclerosis

Blood vessel	**Cardiovascular disease in the family**	
	Yes (%)	**No (%)**
Carotid artery	83.0	17.0
Abdominal artery	70.0	30.0
Femoral artery	70.0	30.0
Iliac artery	50.0	50.0
Coronary artery	52.0	48.0
Mammary artery	62.0	38.0
Total	67.0	33.0

Table 14. Presence of diabetes mellitus type II in the families of patients with different atherosclerotic blood vessels

Blood vessel	Diabetes mellitus type II in the family	
	Yes (%)	**No (%)**
Carotid artery	55.0	45.0
Abdominal artery	40.0	60.0
Femoral artery	30.0	70.0
Iliac artery	50.0	50.0
Coronary artery	24.0	76.0
Mammary artery	50.0	50.0
Total	40.0	60.0

When epidemiologic data were compared based on patients age, as expected, the two age groups with periodontal disease and atherosclerosis showed a statistically significant difference in regard to the incidence of hypertension, diabetes and family history of cardiovascular diseases (Figure 14). All risk factors for development of cardiovascular diseases were more frequent in the group of older patients, and all of them, except smoking, differed statistically between the two groups.

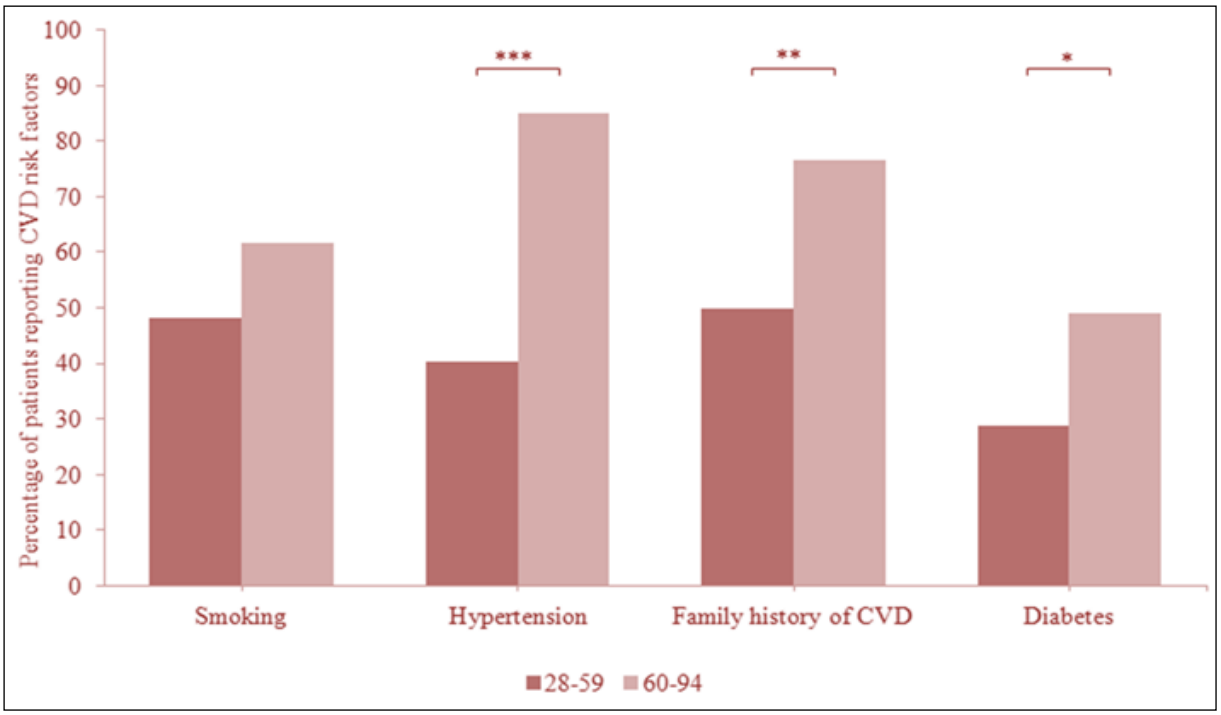


Figure 14. Basic epidemiological and clinical characteristics of the two age groups of patients

4.2. Oral bacteria distribution in the oral cavity and in blood vessels

As already mentioned, 65% of samples of atherosclerotic arteries and 91% of oral samples were positive for at least one of the tested microorganisms. The decreasing frequencies of microorganisms in oral specimens versus artery specimens in the entire patient group, regardless of their age, were as follows: *T. forsythia* (76% vs. 53%), *P. intermedia* (71% vs. 31%), *P. gingivalis* (60% vs. 38%), *A. actinomycetemcomitans* (39% vs. 14%) and *T. denticola* (21% vs. 6%). The distributions of microorganisms in subgingival and atheromatous plaques are given in Figure 15.

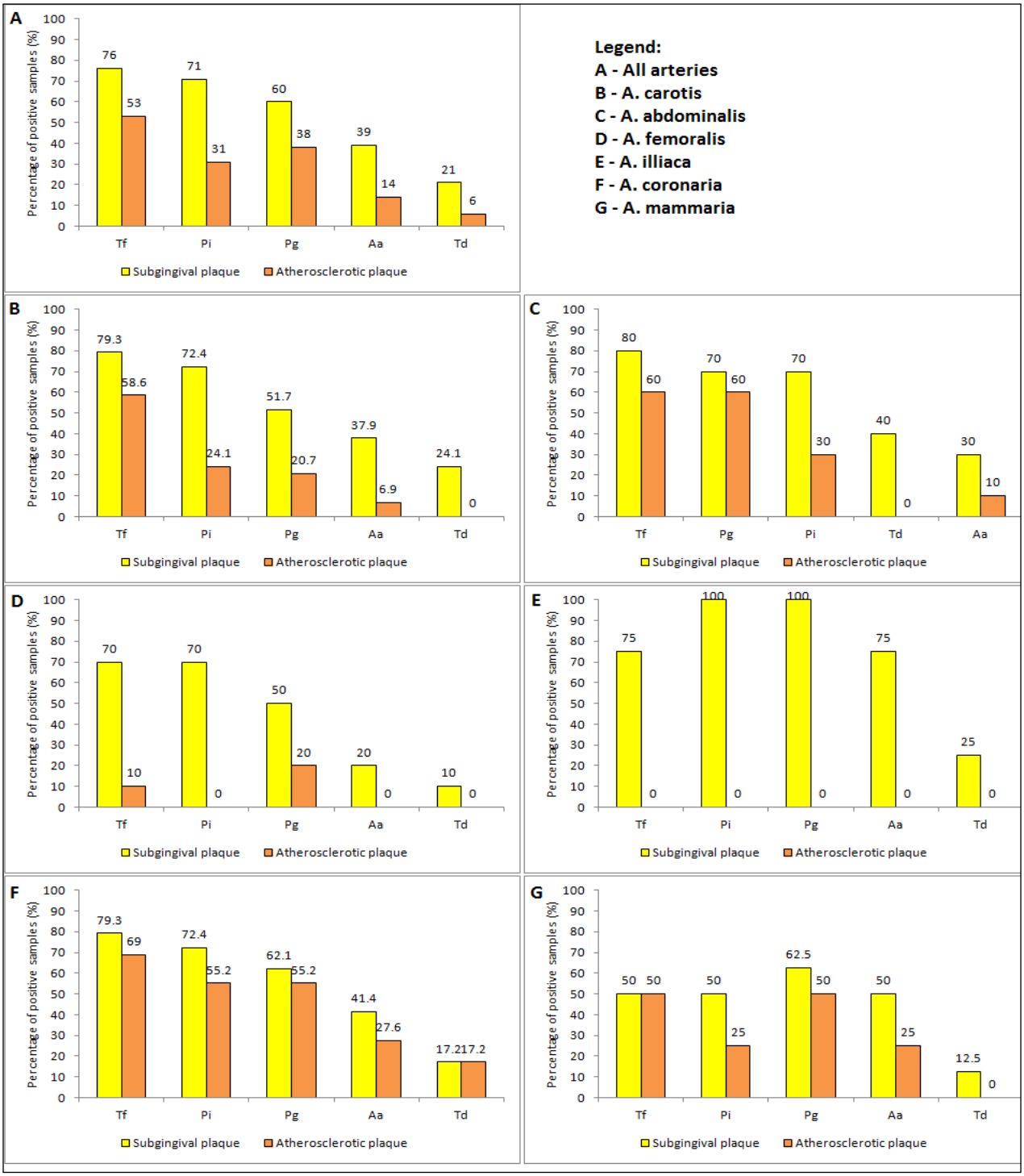


Figure 15. Comparison of microorganisms' incidence in subgingival versus atherosclerotic plaques in the entire sample, regardless of patients' age

The ratio of subgingival versus artery bacteria was as follows: *T. f*orsythia 1.4, *P. gingivalis* 1.6, *P. intermedia* 2.3, *A. actinomycetemcomitans* 2.8 and *T. denticola* 3.5.

Coronary arteries were the blood vessels in which microorganisms were most frequently found, followed by carotid, abdominal and mammary arteries. Oral pathogens were rare in femoral and could not be detected at all in iliac arteries (Figure 16).

Td: *T. denticola*; Aa: *A. actinomycetemcomitans*;
Pg: *P. gingivalis*; Pi: *P. intermedia*; Tf: *T. forsythia*

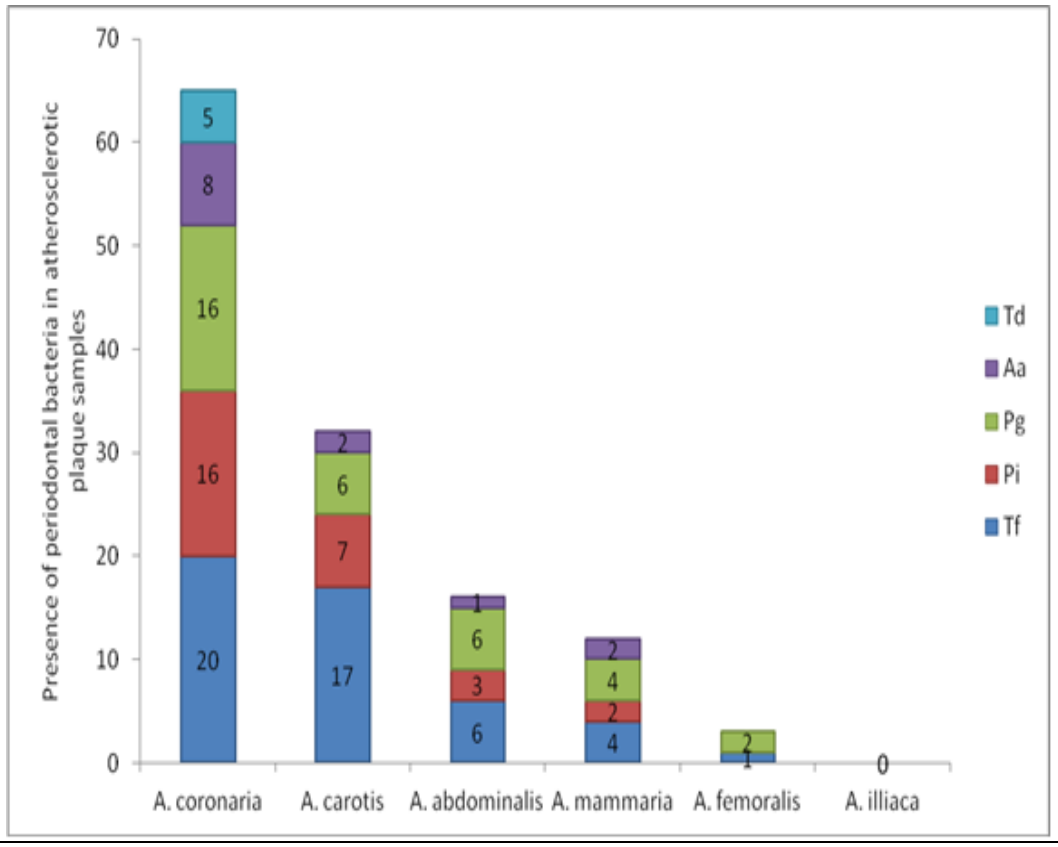
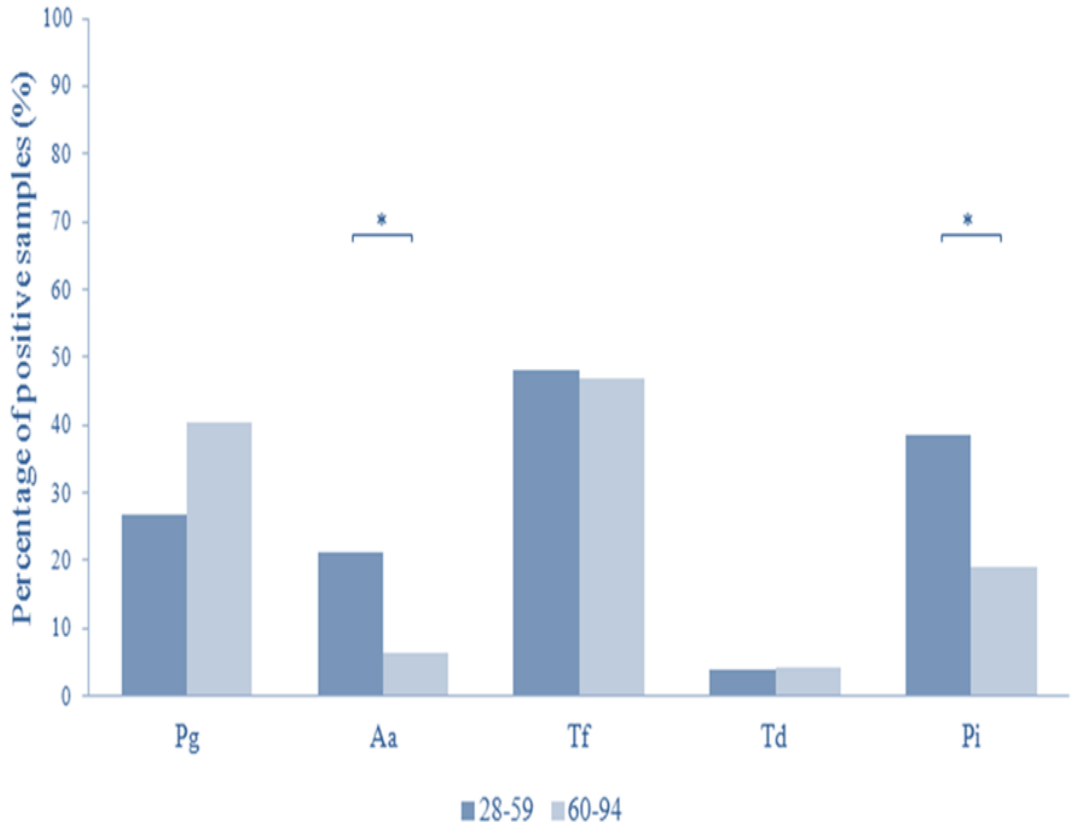


Figure 16. The magnitude of blood vessels colonization by different oral pathogenic bacteria

When focusing on bacterial incidence changes in artery samples in relation to the patients' age, statistically significant difference between younger and older patients were found only for *P. intermedia* and *A. actinomycetemcomitans* (Figure 17).



Pg: *P. gingivalis;* Aa: *A. actinomycetemcomitans;*
Tf: *T. forsythia*; Td: *T. denticola*; Pi: *P. intermedia*

Figure 17. Microorganism incidence in arteries of patients below 60 and over 60 with atherosclerosis

Differences between younger and older patients were also noted when considering the number of different bacterial species per blood vessel, but without reaching statistical significance (Figure 18).

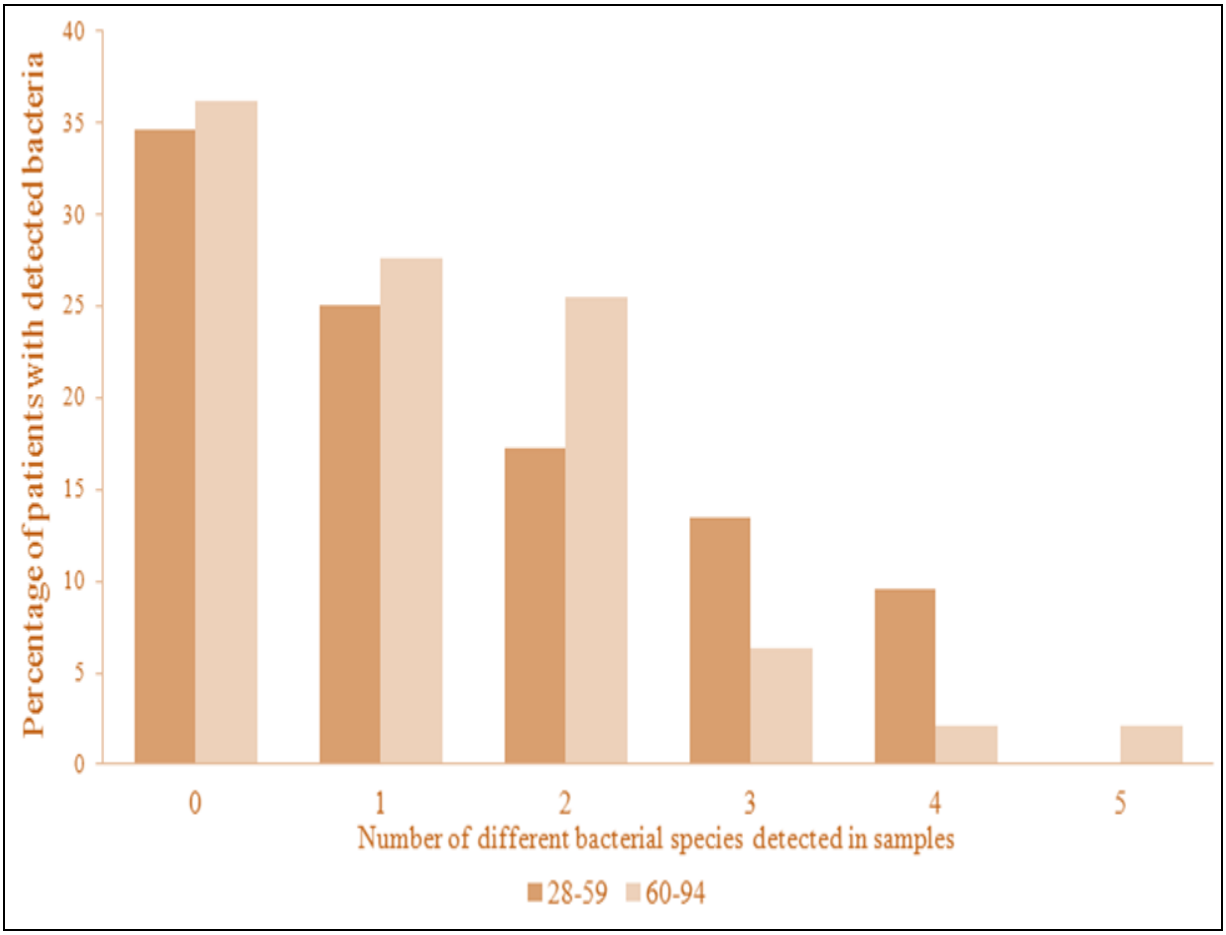


Figure 18. Percentage of artery samples with 0, 1, 2, 3, 4 or 5 different bacterial species in relation to patients' age

When comparing the incidence of bacteria in oral specimens versus blood vessels specimens in relation to patients' age, it appears that the relationships are not dramatically changed (Figure 19).

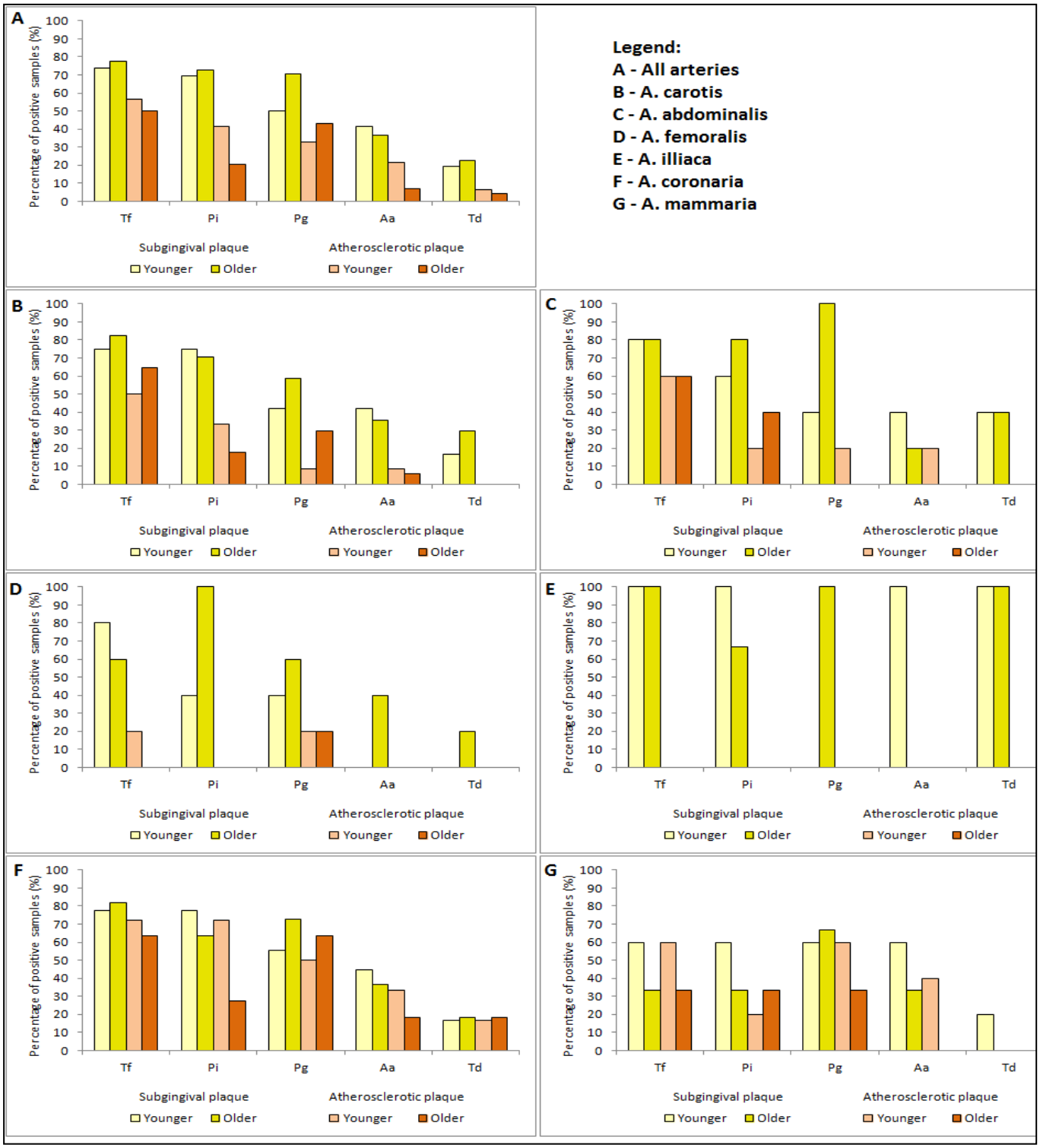


Figure 19. Prevalence of periopathogenic bacteria in subgingival and atherosclerotic plaque samples of younger and older patients; Tf: *T. f*orsythia; Pi: *P. intermedia*; Pg: *P. gingivalis*; Aa: *A. actinomycetemcomitans*; Td: *T. denticola*

4.3. Oral bacteria distribution in thrombi

Only a small number of thrombi were available for the study. Six samples were obtained from patients with myocardial infarction, undergoing catheterization for blood clot removal, while seven samples were obtained from cadavers. In the latter case, samples of atherosclerotic blood vessel and thrombus could be obtained from the same donor, i.e. it was possible to compare the bacterial content in the two types of specimens. This possibility was used not only for qualitative, but also for quantitative comparison of bacterial communities' composition.

When seven atheromas and seven thrombi obtained from cadaver samples were compared, it appeared that bacterial content in thrombi was statistically lower than the content in atheromas (Figures 20 and 21).

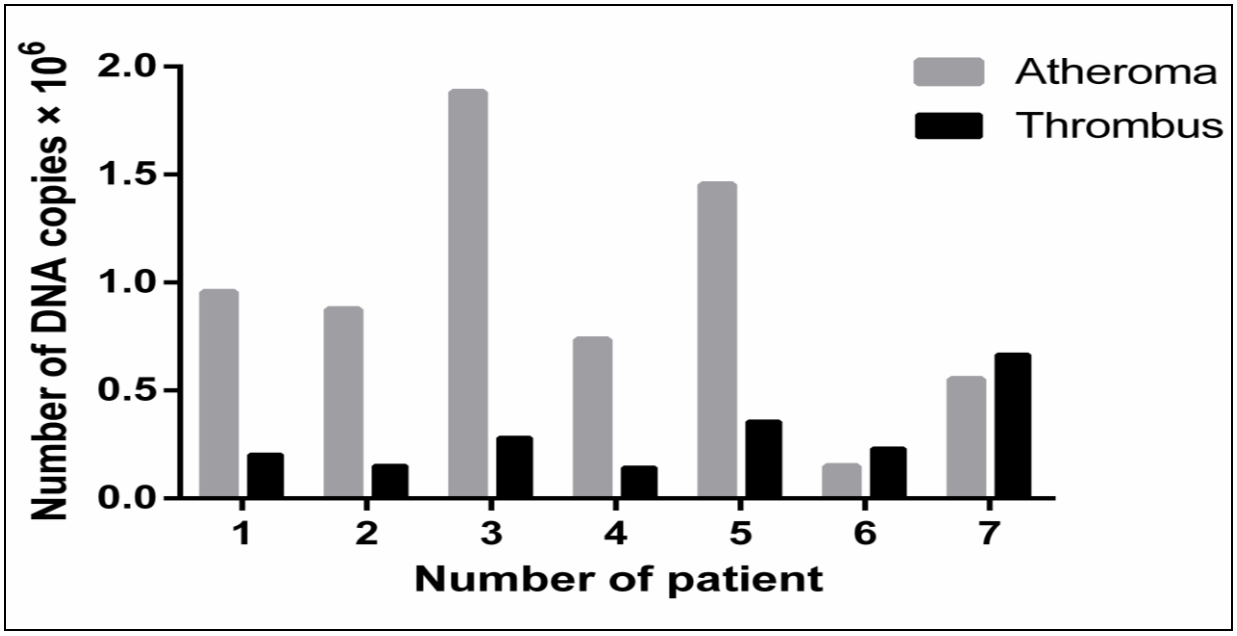


Figure 20. Comparison between bacterial content in atheromas and thrombi obtained from 7 cadavers

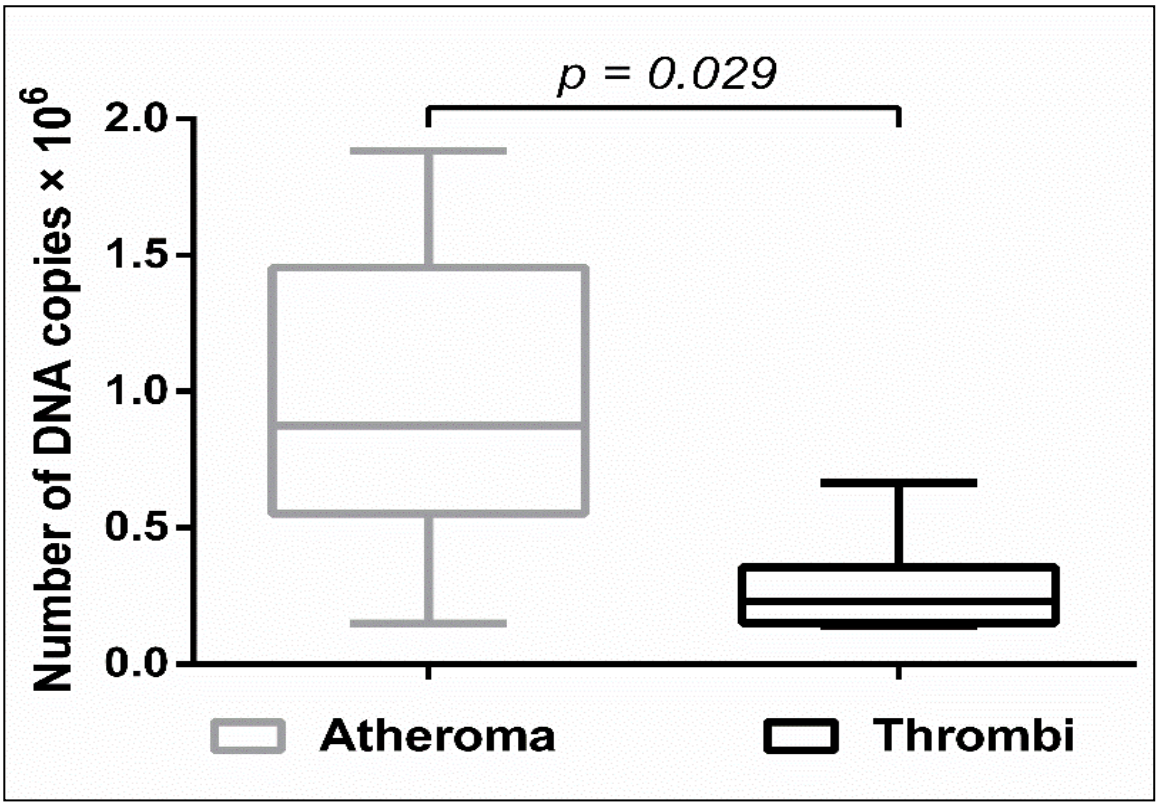


Figure 21. Quantitative analysis of total bacterial content in atheromas versus thrombi

When freshly taken thrombi were also included in the analysis, the picture looked different (Figure 22). Namely, in the fresh thrombi, obviously a higher bacterial content was present.

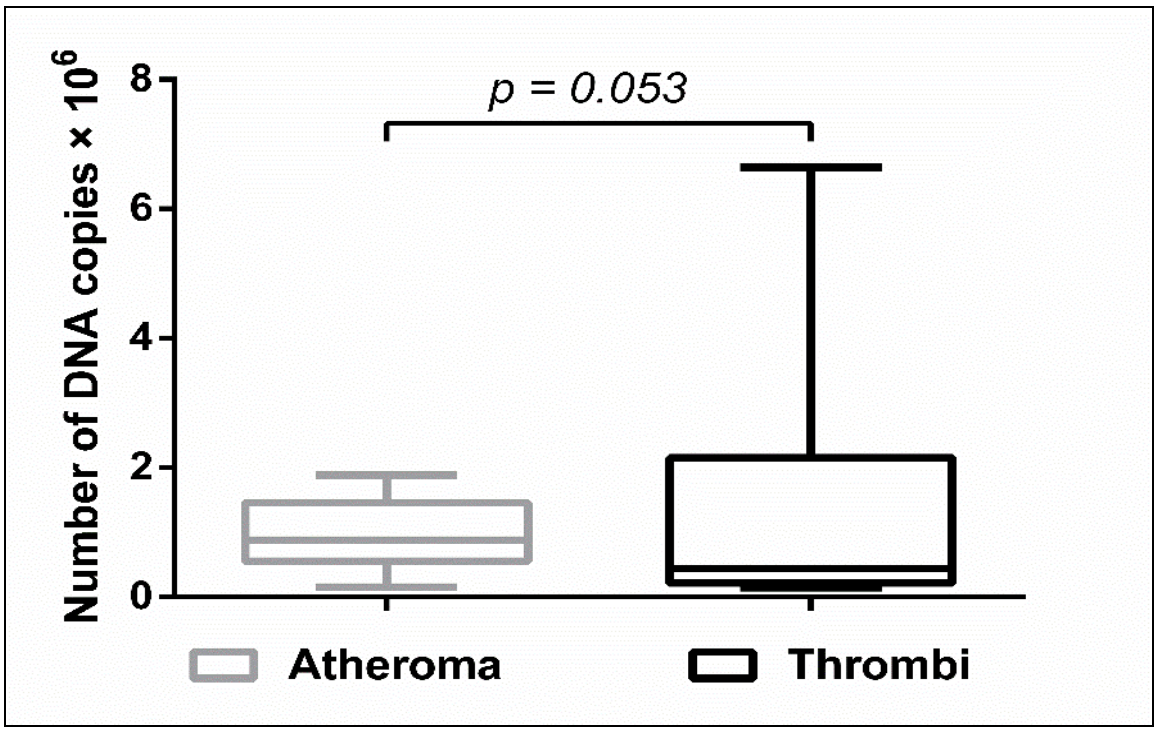


Figure 22. Quantitative analysis of bacterial content in atheromas versus thrombi (all samples)

4.4. Qualitative analysis of oral pathogens and human CMV in atheromas versus thrombi

When comparative analysis of paired atheroma-thrombus samples (from 7 cadavers) was done, it appeared that some bacteria could not be detected at all in thrombi, while some others (*P. gingivalis*) were found only in thrombi (Figure 23).

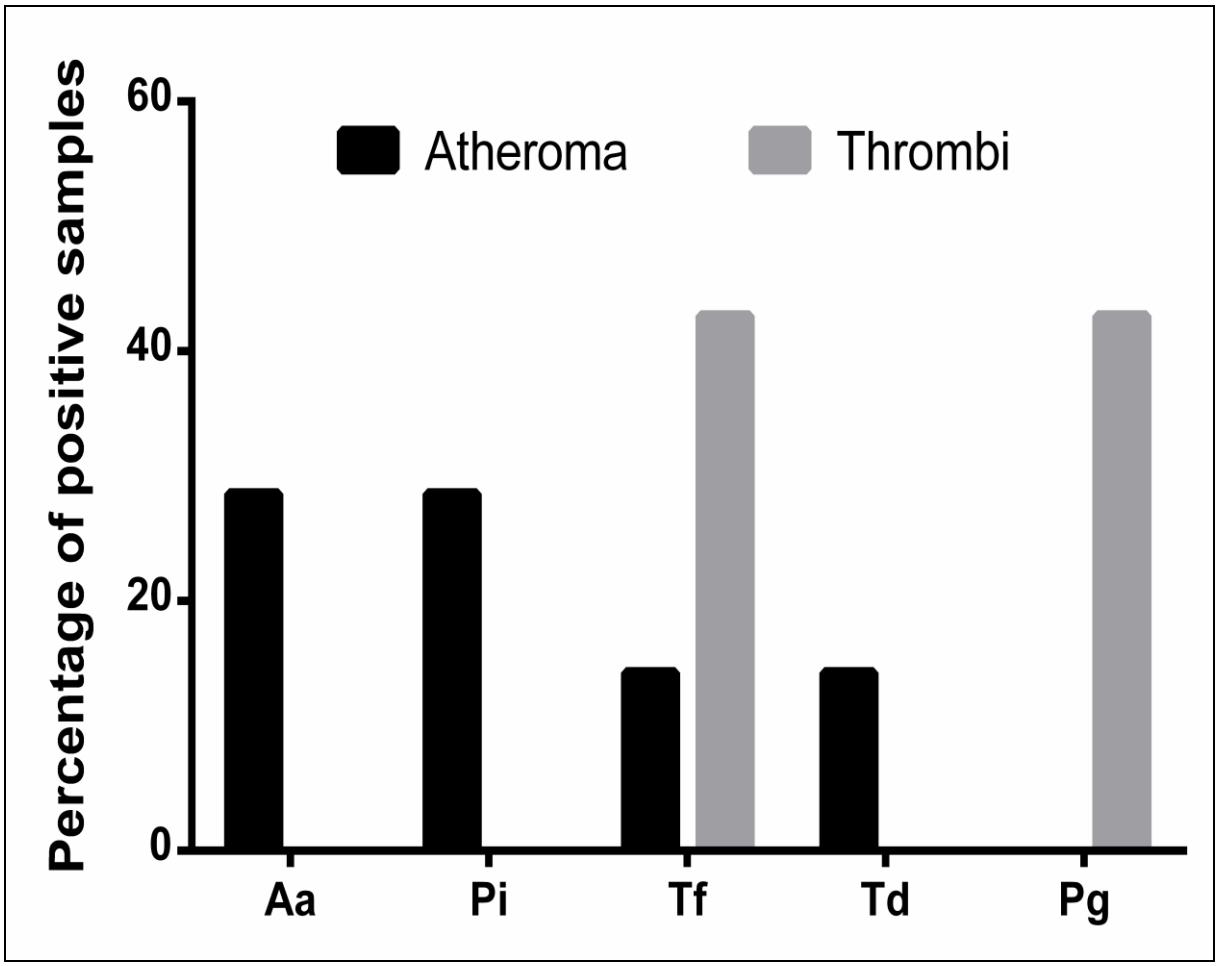


Figure 23. Comparative analysis of bacterial species in atheromas and thrombi in cadaver samples
Aa: *A. actinomycetemcomitans*; Pi: *P. intermedia*; Tf: *T. forsythia*; Td: *T. denticola*; Pg: *P. gingivalis*

The detection of human CMV was also done on a relatively small number of atheromas, in total 23 (16 specimens from patients and 7 specimens from cadavers) and thrombi (13). The results of qualitative analysis for both bacteria and human CMV are given in Figure 24. From this limited analysis it appeared that human CMV was more frequently found in blood clots than in blood vessels, but due to the small sample size, no statistically significant difference could be established.

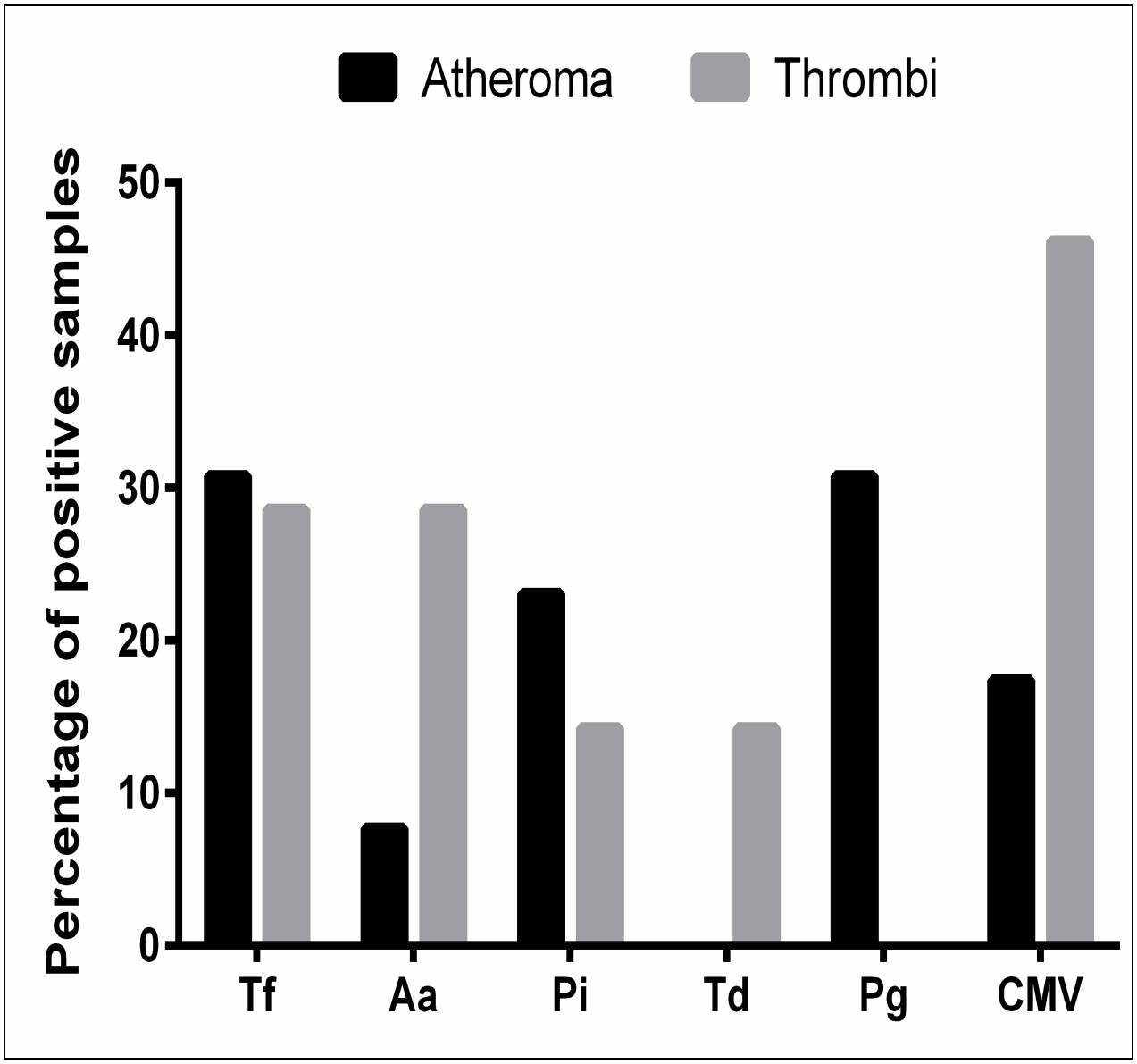


Figure 24. Comparative analysis of bacterial species and human CMV found in a limited number of samples; Tf: *T. forsythia*; Aa: *A. actinomycetemcomitans*; Pi: *P. intermedia*; Td: *T. denticola*; Pg: *P. gingivalis*; CMV: human cytomegalovirus

5. DISCUSSION

The fundamental role of infection and inflammation in the initiation and progression of atherosclerosis is now widely accepte (**Seymour et al. 2007; 2009; Miettinen et al. 1996)**. It must be emphasized that up to 50% of people with CVD, have none of the classic risk factors, such as smoking and alcohol consumption, obesity, high blood pressure and diabetes, implicating that some risk/etiologic factors are still missing. If the problem of high CVD morbidity and mortality has to be tackled, it is essential to uncover all possible risk factors and target all those that are modifiable.

In the last two decades, the cause-effect association between periodontal disease and CVD has been thoroughly assessed **(Inaba & Amano, 2010; Hujoel et al. 2001)**. Namely, it was established that the risk of developing atherosclerosis and myocardial infarction was significantly increased in individuals with severe chronic periodontitis, the most prevalent chronic infection and inflammatory disease in humans worldwide **(Seymour et al. 2007; Valtonen, 1999; Seymour et al. 2007; 2009**)**.** Given that over the past 50 years, there has been a substantial increase in the number of people retaining more of their teeth, this increasingly dentate population will be at even greater risk for periodontal and cardiovascular diseases.

With the present study we aimed at bringing additional information to the complex relationship between periodontal disease and cardiovascular disease, in particular atherosclerosis. The bulk of our study was focused on 100 patients with moderate to severe periodontal disease and atherosclerosis. Atherosclerosis was clinically diagnosed at the Clinic for Vascular and Endovascular Surgery at the Clinical Center Belgrade and at the Institute for Cardiovascular Diseases KBC "Dr Dragiša Mišović" Dedinje in Belgrade. A positive diagnosis was based on clinical findings, coronary angiography and Doppler echosonography. Periodontal disease was diagnosed by routine dental examination. Patients were divided into six groups in relation to the localization of atherosclerosis and into two groups in relation to their age.

Anamnestic and epidemiological analyses showed the following results: there was no statistically significant difference regarding the median age of patients who had different blood vessels affected by atherosclerosis. The highest age span was found among patients with atherosclerosis of carotid arteries. The lowest median age of 54.5 years was established in the group of patients with atherosclerosis of mammary arteries, while the highest was in the group of patients with atherosclerosis of iliac artery (69.5 years). Generally, literature data report on huge variations of the mean age of patients affected by atherosclerosis, depending on patients' ethnical background and whether they live in high or low income countries, as thoroughly stressed in the Introduction section.

The majority of our patients had a secondary degree of education, which is in agreement with epidemiological data presented in some other studies on atherosclerosis and CVD **(Eng et al. 2015)**, but this relatively high level of education had no impact on oral pathogenic microorganisms' presence.

No statistically significant difference was noted between the smoking status of patients belonging to different study subgroups, nor could a difference be established between smokers and non-smokers in terms of microorganisms' presence. Some authors indicated smoking as a significant confounding factor influencing the seemingly present association between periodontitis and CVD **(Chukkapalli et al. 2015; Hujoel et al. 2002)**. This is supported by analyses which include adjustments for some smoking-related variables such as the number of cigarettes per year, time after stopping to smoke, passive smoking, etc. **(Chukkapalli et al. 2015; Nasry et al. 2006; Costa et al. 2013)**. However, association between periodontitis and CVD was also recurrently described in studies on never-smokers **(Chukkapalli et al. 2015; Okoro et al. 2005),** leaving this question opened for future analyses.

Similarly, no consistent association could be established between the presence of microorganisms and alcohol consumption in the different study subgroups. This can be explained, either by the

small sample size, or by the fact that patients' answers, especially with regard to questions about drinking, are not reliable. And again, no correlation was found between different groups regarding oral hygiene.

There was a huge variation in the frequency of hypertension in patients with different blood vessels affected by atherosclerosis (from 100% of hypertensive patients in the group with iliac artery atherosclerosis to only 30% of hypertensive patients in the group of femoral artery atherosclerosis), but without statistically significant difference, between the groups. The majority of patients had a family history of cardiovascular disease (from 50% in the group with iliac artery atherosclerosis, to 83% in the group with carotid artery atherosclerosis). Diabetes mellitus was less represented in the family history (from 24% in patients with coronary artery atherosclerosis, to 55% in patients with carotid artery atherosclerosis). It must be emphasized that there is a bidirectional relationship between periodontitis and systemic diseases, i.e. patients with some systemic disease, such as CVD or diabetes are more prone to periodontitis and often the cause-effect is not easy to establish. The influence of diabetes mellitus on periodontal disease is not fully explored yet. Some studies in adults have found no association between periodontal disease and diabetes. On the other hand, some have shown an increased susceptibility to periodontal disease in adults with diabetes mellitus **(Thorstensson & Hugoson 1993)**. In other words, the complex relationship existing between CVD/diabetes and periodontal disease needs further clarifications.

When the two age groups were taken into consideration, regardless of the affected artery, a statistically significant difference appeared in the frequency of hypertension and family history of cardiovascular disease, "in favor" of the older age group, which was a rather expected finding.

The relationship between periodontal disease and CVD is influenced by age, with a stronger association in patients below 60-65 yrs compared to patients 60-65 **(Chukkapalli et al. 2015; Sim et al. 2008).** At the same time, it was also established that individuals with treated periodontal disease older than 60 years have increased risk of long-term detrimental cardiovascular events compared with subjects below 60 years. The association remained after adjustment for other potential CVD-related confounders such as gender, hypertension, diabetes and hyperlipidemia **(Chukkapalli et al. 2015; Chou et al. 2015)**.

In the present study, as much as 91% of subgingival plaques tested positive for the studied microorganisms, while 65% of atherosclerotic arteries were positive for at least one of the oral pathogens. These findings support the idea that periodontal pathogens enter the circulation, causing transient bacteremia, and then lodge in diseased blood vessel walls.

The PCR analysis of both oral cavity and atherosclerotic blood vessels' tissue samples showed a high prevalence of the following oral pathogens: *Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola* and *Aggregatibacter actinomycetemcomitans*. This result is in general agreement with a number of previous reports dealing with periodontal disease and atherosclerosis **(Pucar et al. 2007; Kebschull et al. 2010; Bartova et al. 2014; Hussain et al. 2015; Bale et al. 2016)**.

The most prevalent bacterium in both oral and blood vessel samples was *T. forsythia*, and the least prevalent was *T. denticola*. The decreasing frequencies of microorganisms in oral specimens were: *T. forsythia* (76%), *P. intermedia* (71%), *P. gingivalis* (60%), *A. actinomycetemcomitans* (39%) and *T. denticola* (21%), whereas in atherotic samples the frequencies were lower and the decreasing order was somewhat different compared to the oral cavity: *T. forsythia* (53%), *P. gingivalis* (38%), *P. intermedia* (31%), *A. actinomycetemcomitans* (14%) and *T. denticola* (6%). Although there is a consensus regarding the most prevalent pathogenic microorganisms detected both in subgingival and atheromatous plaque, the rate of their detection (presence) may vary considerably from one study to another. For instance, Mahendra et al. reported the following incidence of bacteria, rather opposite to ours (in the subgingival and atheroma plaque): *T. denticola* 66% and 51%, *P. gingivalis*

64% and 45%, *T. forsythia* 43% and 31%, *P. intermedia* 27% and 23%, *A. actinomycetemcomitans* 2% and 0% **(Mahendra et al, 2015).** In the study of **Zaremba et al. (2007)**, *P. gingivalis* and *T. denticola* were the two most frequently detected microorganisms. Interestingly, in the study of Romano et al. percentages of bacteria similar to ours were determined in oral samples, but these authors did not detect the presence of any of the five microorganisms in 21 atheromas **(Romano et al. 2007)**.

A noteworthy finding of the present study was that different bacteria showed different affinity for blood vessel colonization (or different capacity to invade them). Namely, as judged from the ratio of bacteria present in the subgingival plaque versus bacteria in the arteries, it appeared that *T. forsythia* had the highest propensity to colonize artery walls, while *T. denticola* had the lowest. This ratio for different bacteria was the following: *T. forsythia* 1.4, *P. gingivalis* 1.6, *P. intermedia* 2.3, *A. actinomycetemcomitans* 2.8 and *T. denticola* 3.5 **(Kannosh et al. 2018)**.

Regarding the process of aging and it's rather expected effects on bacteria distribution, only the incidence of *P. gingivalis* increased in arteries with patients' age. *T. denticola* and *T. forsythensis* incidence remained unchanged, while the incidence of *A. actinomycetemcomitans* and *P. intermedia* showed a statistically significant decrease in the older group of patients. A comparable decrease for *A. actinomycetemcomitans* with aging has been previously described **(Andjelkovic et al. 2017).**

When considering blood vessels in relation to their susceptibility to be colonized, coronary arteries were the blood vessels in which microorganisms were most frequently found, followed by carotid, abdominal and mammary arteries. Oral pathogens were rare in femoral and could not be detected at all in illiac arteries. This indicates that bacterial colonization mirrors the distance of a given artery from the mouth, which is the primary bacterial reservoir. Again, in the literature a remarkable heterogeneity of results can be found. For instance, Szulc et al. found *P. gingivalis* in less than 10% of coronary arteries, while in the carotid arteries almost 50% of their samples were positive for this microorganism **(Szulc et al. 2015).** Pucar et al., on the contrary found 53% of coronary arteries to be positive for the presence of *P. gingivalis*, 27% positive for *A. actinomycetemcomitans*, 33% positive for *P. intermedia* and 13% for *T. forsythensis*. Interestingly in their study 0% of mammary arteries were affected by oral pathogens, a finding that is in contrast with our results. They also detected a high percentage of CMV in their samples (67%), which was not the case in the present study in which only 17% of atheromas were positive **(Pucar et al, 2007).** Taylor-Robinson et al found oral bacteria in specimens from the iliac, mammary and coronary arteries and aorta, but the incidence of oral pathogens was rather low. They found approximately 20% of specimens to be positive *for A. actinomycetemcomitans* and only 9% for *P. intermedia*. Different data were obtained by Haraszthy et al: 30% of the surgical specimens were positive for *B. forsythia*, 26% for *P. gingivalis*, 18% for *A. actinomycetemcomitans*, and 14% for *P. intermedia*. In the atheromas positive for periodontal pathogens, more than one species were most often detected (59%), a finding close to ours **(Haraszthy et al. 2000).** This group however found a higher percentage of human CMV (38%) in their atheroma samples than we did (17%). In a very recent study, statistical difference was observed for the prevalence of 16S rRNA of *P. gingivalis, T. forsythia, T. denticola* and *P. nigrescens* both in subgingival and atheroma plaque of patients with ischemic heart disease compared to controls. In addition, co-occurrence of nine different bacteria in subgingival and atheroma plaque was observed in patients with ischemic heart disease. A similar co-occurrence was not seen in patients with chronic periodontitis but without systemic disease. The authors concluded that their study correlates periodontal bacterial co-occurrence to atherosclerosis" **(Mahalakshmi et al. 2017)**.

Similarly to data related to the incidence of periopathogenic bacteria, important variations of human CMV detection in blood vessels of patients with atherosclerosis were also noted. In the Chinese Han population, 60% of patients with carotid atherosclerosis and stroke were positive for HCMV, when PCR was used **(Yi et al. 2008)**. The same group found lower levels of viral infection when

applying other techniques (34% found by immunohistochemistry and 40% found by *in situ* hybridization). Virok et al found human CMV in 50% of their carotid samples, either alone or in combination with *Chlamydia pneumoniae* **(Virok et al. 2006).** Pucar et al also detected a high percentage of CMV in their samples (67%), which was not the case in the present study in which only 17% of atheromas were positive **(Pucar et al. 2007)**. Haraszthy et al, in a group of Hungarian patients with atherosclerosis, detected human CMV in 38% of their atheroma samples **(Haraszthy et al, 2000)**. On the other hand, Tremolada et al did not find CMV nor SV1 in any of their 17 samples of atherosclerotic plaques of carotid arteries **(Tremoladai et al. 2011).** A recent meta-analysis, involving 30 studies and 3228 patients with atherosclerosis, showed the presence of viral DNA in 34% of cases **(Jia et al. 2017).** The authors concluded that human CMV is significantly related to increased risk of atherosclerosis.

In the present study, human CMV was also assessed in thrombi of patients with myocardial infarction and in this specific biological material a much higher percentage of the virus was detected than in atheromas. Namely, 50% of thrombi contained CMV. This finding is in line with the evidence that acute-phase ~~H~~CMV infection is related to thrombogenesis and thrombosis **(Rahbar and Söderberg-Nauclér, 2005)**. Infected endothelial cells, a reservoir of CMV, recruit naive monocytes from the blood stream and allow virus transfer to migrating monocytes, which will lead to its dissemination. At the same time the virus will enhance the expression of endothelial adhesion molecules and thrombin production, resulting in clotting impairment **(Popovic et al. 2012)**.

Besides social, geographical and ethnical explanations for the huge data variations, one must also take into consideration the possibility that the methodology used for bacteria detection might influence the accuracy of the findings and, importantly, the biological material used for oral pathogen detection, as well. Two main techniques are usually applied: cultures and PCR. PCR is considered to be the technique of choice for overcoming the restrictions related to culturing bacteria in terms of rapidity and sensitivity, since even one copy of the DNA target present in the clinical specimen can be identified **(Sanz et al. 2004)**. PCR primers directed against conserved regions in the 16S rRNA gene are designed to specifically amplify either Gram-positive or Gram-negative bacteria; therefore, under appropriate conditions, PCR also exhibits very high specificity and has a superior performance compared with classical microbiological approaches **(Santos et al. 2004).** Standard diagnosis of bacterial infection, which depends on bacteria growth in culture, usually requires at least 12 to 72 h for detection and many anaerobes are difficult to grow. Molecular genetics methods for bacterial DNA detection are much faster and have shown great efficacy and sensitivity (greater than immunostaining). In particular, they are very useful for detection of strictly anaerobic bacteria. Even if the samples contain dead bacteria, PCR can still be applied as cell viability is not an imperative, i.e. because bacterial DNA is still in the sample **(Garibyan & Avashia, 2013)**. There are also different variants of conventional PCR that are used in fundamental or applied oral biology **(Leys et al. 1994**). For example, DNA microarray analysis is used for a fast semi-quantitative analysis of the main periodontal pathogens **(Topcuoglu & Kulekci, 2015)**. Another technique, called viability PCR or vPCR, relies on cell viability and cell envelope impermeability **(Nocker & Camper, 2008).** In this method propidium monoazide is added in the PCR mixture. This substance selectively enters dead bacteria, intercalates with their DNA and consequently blocks PCR. Therefore, only microorganisms that are alive will be detected. In addition, another matter of debate is which type of sample is the best and most convenient to use in order to get reliable information regarding the presence of oral pathogens (buccal swabs, saliva, subgingival/supragingival plaque). In their study Sakamoto et al compared two types of samples (saliva and subgingival plaque samples) and three methods (cell culture, conventional PCR and qPCR) for the identification and quantification of periodontal microorganisms. The results indicated that saliva in combination with qPCR gave good results regarding the detection and quantification of periodontal bacteria in the oral cavity **(Sakamoto et al. 2001)**.

Although all PCR based techniques have some limitations, with the possibility, although remote, of contaminations, cross-reactions, false positive results, false negative results, etc., they undeniably revolutionized our knowledge on periodontal pathogens **(Kotsilkov et al. 2015)**. Recently, commercial diagnostic tests using multiplex PCR (e.g., MyPerioPath®, MicroDent® Test, etc.) have been introduced for the assessment of microorganisms in subgingival plaque samples, allowing for a better prevention program and the development of individualized treatment **(Do, Devine & Marsh 2013)**.

In conclusion, our results support the idea that exposure to periodontal microorganisms and concomitant inflammation play a role in the etiology of atherosclerosis and myocardial infarction. However, it must be emphasized that our study covered only one point in time. Therefore, in order to more accurately perceive the role of periodontal pathogens in the development of those diseases, precise measurements of the intensity of periodontal disease, frequency and length of bacteremia and duration of exposure to inflammatory mediators coming from the periodontium, all in a wider time window, should be conducted.

6. CONCLUSIONS

- Oral pathogenic bacteria were abundant in both subgingival plaque and blood vessels of patients with atherosclerosis, with an overall ratio of 91% versus 65% in the two types of samples

- The most prevalent bacteria in both types of biological samples was *T. forsythia* and the least prevalent was *T. denticola*

- With aging, the incidence of bacteria changed slightly. The decreasing order of bacterial incidence in younger patients was the following: *T. forsythia* > ***P. intermedia*** > ***P. gingivalis*** > *A. actinomycetemcomitans* > *T. denticola* while in the elderly patients it was: *T. forsythia* > ***P. gingivalis*** > ***P. intermedia*** > *A. actinomycetemcomitans* > *T. denticola*

- Interestingly, there was no statistically significant difference in the total incidence of microorganisms between the two age groups

- The decreasing order of atherosclerotic bloods vessels according to incidence of oral pathogens was the following: coronary artery > carotid artery > abdominal artery > mammary artery > femoral artery > iliac artery

- Bacterial quantification performed in a limited number of samples showed that there was no significant difference in the total bacterial content between thrombi and atheromas

- The qualitative analysis, however showed slight differences in the presence of different bacterial species

- Importantly, human CMV appeared to be much more frequent in thrombi than in atheromas

7. REFERENCES

Alberti A, Romano C, Falco M, Cali F, Schinocca P, Galesi O, Spalletta A, Di Benedetto D, Fichera M, 2007. 1.5 Mb de novo 22q11. 21 microduplication in a patient with cognitive deficits and dysmorphic facial features. *Clinical genetics*. 71(2):177-82.

Amin MM, Stowell JD, Hendley W, Garcia P, Schmid DS, Cannon MJ, Dollard SC, 2018. *CMV* on surfaces in homes with young children: results of PCR and viral culture testing. *BMC infectious diseases.* 18(1):391.

Anand PS, Kamath KP, Anil S, 2014. Role of dental plaque, saliva and periodontal disease in *Helicobacter pylori* infection. *World journal of gastroenterology: WJG*. 20(19):5639.

Andjelkovic M, Sojic LT, Lemic AM, Nikolic N, Kannosh IY, Milasin J, 2017. Does the prevalence of periodontal pathogens change in elderly edentulous patients after complete denture treatment?. *Journal of Prosthodontics.* 26(5):364-9.

Ansai T, Yamamoto E, Awano S, Yu W, Turner AJ, Takehara T, 2002. Effects of periodontopathic bacteria on the expression of endothelin-1 in gingival epithelial cells in adult periodontitis. Clin Sci (Lond) 103 (s2002): 327S-331S.

Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR, 2005. Basic principles of real-time quantitative PCR. *Expert review of molecular diagnostics*. 5(2):209-19.

Bale BF, Doneen AL, Vigerust DJ, 2017. High-risk periodontal pathogens contribute to the pathogenesis of atherosclerosis. *Postgraduate medical journal*. 93(1098):215-20.

Bartova J, Sommerova P, Lyuya-Mi Y, Mysak J, Prochazkova J, Duskova J, Janatova T, Podzimek S, 2014. Periodontitis as a risk factor of atherosclerosis. *Journal of immunology research*. 2014.

Batty GD, Jung KJ, Mok Y, Lee SJ, Back JH, Lee S, Jee SH, 2018. Oral health and later coronary heart disease: Cohort study of one million people. *European journal of preventive cardiology*. 25(6):598-605.

Beck JD, Slade G, Offenbacher S, 2000. Oral disease, cardiovascular disease and systemic inflammation. *Periodontology 2000*. 23(1):110-20.

Becker AE, de Boer OJ, van der Wal AC, 2001. The role of inflammation and infection in coronary artery disease. *Annual review of medicine*. 52(1):289-97.

Bhagat K, Moss R, Collier J, Vallance P, 1996. Endothelial "stunning" following a brief exposure to endotoxin: a mechanism to link infection and infarction?. *Cardiovascular research.* 32(5):822-9.

Bostanci N, Belibasakis GN, 2012. *Porphyromonas gingivalis*: an invasive and evasive opportunistic oral pathogen. *FEMS microbiology letters*. 333(1):1-9.

Bovet P, Paccaud F, 2011. Cardiovascular disease and the changing face of global public health: a focus on low and middle income countries. *Public Health Reviews*. 33(2):397.

Braham PH, Moncla BJ, 1992. Rapid presumptive identification and further characterization of *Bacteroides forsythus*. *Journal of clinical microbiology*. 30(3):649-54.

Brajović G, Popović B, Puletić M, Kostić M, Milašin J, 2016. Estimation of total bacteria by real-time PCR in patients with periodontal disease. *Srpski arhiv za celokupno lekarstvo*. 144(1-2):10-4.

Buhlin K, Hultin M, Norderyd O, Persson L, Pockley AG, Rabe P, Klinge B, Gustafsson A, 2009. Risk factors for atherosclerosis in cases with severe periodontitis. *Journal of clinical periodontology*. 36(7):541-9.

Caesar R, Fåk F, Bäckhed F, 2010. Effects of gut microbiota on obesity and atherosclerosis via modulation of inflammation and lipid metabolism. *Journal of internal medicine*. 268(4):320-8.

Choi EK, Park SA, Oh WM, Kang HC, Kuramitsu HK, Kim BG, Kang IC, 2005. Mechanisms of *Porphyromonas gingivalis*-induced monocyte chemoattractant protein-1 expression in endothelial cells. *FEMS Immunology & Medical Microbiology*. 44(1):51-8.

Chou SH, Tung YC, Lin YS, Wu LS, Lin CP, Liou EJ, Chang CJ, Kung S, Chu PH, 2015. Major adverse cardiovascular events in treated periodontitis: a population-based follow-up study from Taiwan. *PLoS One*. 10(6):e0130807.

Chukkapalli SS, Rivera-Kweh MF, Velsko IM, Chen H, Zheng D, Bhattacharyya I, Gangula PR, Lucas AR, Kesavalu L, 2015. Chronic oral infection with major periodontal bacteria *Tannerella forsythia* modulates systemic atherosclerosis risk factors and inflammatory markers. *Pathogens and disease*. 73(3).

Costa FO, Cota LO, Lages EJ, Cyrino RM, Oliveira AM, Oliveira PA, Cortelli JR, 2013. Associations of duration of smoking cessation and cumulative smoking exposure with periodontitis. *Journal of oral science*. 55(3):245-53.

Darveau RP, 2009. The oral microbial consortium's interaction with the periodontal innate defense system. *DNA and cell biology*. 28(8):389-95.

Davé S, Van Dyke TE, 2008. The link between periodontal disease and cardiovascular disease is probably inflammation. *Oral diseases*. 14(2):95-101.

Dashper SG, Seers CA, Tan KH, Reynolds EC, 2011. Virulence factors of the oral spirochete *Treponema denticola*. *Journal of dental research*. 90(6):691-703.

Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs Jr DR, Sacco RL, Papapanou PN, 2005. Periodontal microbiota and carotid intima-media thickness: the Oral Infections and Vascular Disease Epidemiology Study (INVEST). *Circulation*. 111(5):576-82.

Do T, Devine D, Marsh PD, 2013. Oral biofilms: molecular analysis, challenges, and future prospects in dental diagnostics. *Clinical, cosmetic and investigational dentistry*. 5:11.

Doungudomdacha S, Rawlinson A, Douglas CW, 2000. Enumeration of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* in subgingival plaque samples by a quantitative-competitive PCR method. *Journal of medical microbiology*. 49(10):861-74.

Du Y, Zhang G, Liu Z, 2018. *Human cytomegalovirus* infection and coronary heart disease: a systematic review. *Virology journal.* 15(1):31

Dzink JL, Socransky SS, Haffajee AD, 1988. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *Journal of clinical periodontology*. 15(5):316-23.

Ebersole JL, Machen RL, Steffen MJ, Willmann DE, 1997. Systemic acute- phase reactants, C- reactive protein and haptoglobin, in adult periodontitis. *Clinical & Experimental Immunology*. 107(2):347-52.

Ellis JE, Heuser R, Missan DS, Martinez D, Heningburg A, Shabilla M, Schwartz R, Fry S, 2017. Evidence for polymicrobial communities in explanted vascular filters and atheroma debris. *Molecular and cellular probes*. 33:65-77.

Eng J, McClelland RL, Gomes AS, Hundley WG, Cheng S, Wu CO, Carr JJ, Shea S, Bluemke DA, Lima JA, 2015. Adverse left ventricular remodeling and age assessed with cardiac MR imaging: the multi-ethnic study of atherosclerosis. *Radiology*. 278(3):714-22.

Espinola-Klein C, Rupprecht HJ, Blankenberg S, Bickel C, Kopp H, Victor A, Hafner G, Prellwitz W, Schlumberger W, Meyer J, 2002. Impact of infectious burden on progression of carotid atherosclerosis. *Stroke*. 33(11):2581-6.

Fabricant CG, Fabricant J, Litrenta MM, Minick CR, 1978. Virus-induced atherosclerosis. *Journal of Experimental Medicine*. 148(1):335-40.

Fraga D, Meulia T, Fenster S, 2008. Real- time PCR. *Current protocols essential laboratory techniques*.(1):10-3.

Garibyan L, Avashia N, 2013. Polymerase Chain Reaction. *Journal of Investigative Dermatology*, 133(3):1–4.

Gholizadeh P, Pormohammad A, Eslami H, Shokouhi B, Fakhrzadeh V, Kafil HS, 2017. Oral pathogenesis of *Aggregatibacter actinomycetemcomitans*. *Microbial pathogenesis*. 113:303-11.

Gibbons RJ, 1989. Bacterial adhesion to oral tissues: a model for infectious diseases. *Journal of dental research*. 68(5):750-60.

Ginzinger DG, 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Experimental hematology*. 30(6):503-12.

Grenier D, 1995. Characterization of the trypsin-like activity of *Bacteroides forsythus*. *Microbiology*. 141(4):921-6.

Hajishengallis G, 2011. Immune evasion strategies of *Porphyromonas gingivalis*. *Journal of oral biosciences*. 53(3):233-40.

Hajishengallis G, Darveau RP, Curtis MA, 2012. The keystone-pathogen hypothesis. *Nature Reviews Microbiology*. 10(10):717.

Hajishengallis G, Lamont RJ, 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Molecular oral microbiology*. 27(6):409-19.

Hasebe A, Yoshimura A, Into T, Kataoka H, Tanaka S, Arakawa S, Ishikura H, Golenbock DT, Sugaya T, Tsuchida N, Kawanami M, 2004. Biological activities of *Bacteroides forsythus* lipoproteins and their possible pathological roles in periodontal disease. *Infection and immunity*. 72(3):1318-25.

Henry LG, McKenzie RM, Robles A, Fletcher HM, 2012. Oxidative stress resistance in *Porphyromonas gingivalis*. *Future microbiology*. 7(4):497-512.

Higashi Y, Goto C, Jitsuiki D, Umemura T, Nishioka K, Hidaka T, Takemoto H, Nakamura S, Soga J, Chayama K, Yoshizumi M, Taguchi A., 2008. Periodontal infection is associated with endothelial dysfunction in healthy subjects and hypertensive patients. *Hypertension*. 51(2):446-53.

Honma K, Kuramitsu HK, Genco RJ, Sharma A, 2001. Development of a Gene Inactivation System for *Bacteroides forsythus*: Construction and Characterization of a BspA Mutant. *Infection and immunity*. 69(7):4686-90.

Hughes CV, Malki G, Loo CY, Tanner AC, Ganeshkumar N., 2003. Cloning and expression of alpha-D-glucosidase and N-acetyl-beta-glucosaminidase from the periodontal pathogen, *Tannerella forsythensis* (*Bacteroides forsythus*). *Oral Microbiol Immunol.* 18(5):309-12.

Hujoel PP, Drangsholt M, Spiekerman C, Derouen TA, 2001. Examining the link between coronary heart disease and the elimination of chronic dental infections. *The Journal of the American Dental Association*. 132(7):883-9.

Hussain M, Stover CM, Dupont A, 2015. *P. gingivalis* in periodontal disease and atherosclerosis–scenes of action for antimicrobial peptides and complement. *Frontiers in immunology*. 6:45.

Inaba H, Amano A, 2010. Roles of oral bacteria in cardiovascular diseases—from molecular mechanisms to clinical cases: Implication of periodontal diseases in development of systemic diseases. *Journal of pharmacological sciences*. 113(2):103-9.

Inagaki S, Kimizuka R, Kokubu E, Saito A, Ishihara K, 2016. *Treponema denticola* invasion into human gingival epithelial cells. *Microbial pathogenesis*. 94:104-11.

Ishihara K, Nabuchi A, Ito R, Miyachi K, Kuramitsu HK, Okuda K, 2004. Correlation between detection rates of periodontopathic bacterial DNA in carotid coronary stenotic artery plaque and in dental plaque samples. *Journal of clinical microbiology*. 42(3):1313-5.

Iwai T, Inoue Y, Umeda M, Huang Y, Kurihara N, Koike M, Ishikawa I, 2005. Oral bacteria in the occluded arteries of patients with Buerger disease. *Journal of vascular surgery*. 42(1):107-15.

Jakovljevic A, Andric M, Knezevic A, Milicic B, Beljic-Ivanovic K, Perunovic N, Nikolic N, Milasin J, 2017. Herpesviral-bacterial co-infection in mandibular third molar pericoronitis. *Clinical oral investigations*. 21(5):1639-46.

Jia YJ, Liu J, Han FF, Wan ZR, Gong LL, Liu H, Zhang W, Wardell T, Lv YL, Liu LH, 2017. *Cytomegalovirus* infection and atherosclerosis risk: A meta- analysis. *Journal of medical virology*. 89(12):2196-206.

Jorth P, Turner KH, Gumus P, Nizam N, Buduneli N, Whiteley M, 2014. Metatranscriptomics of the human oral microbiome during health and disease. *MBio*. 5(2):e01012-14.

Kannosh I, Staletovic D, Toljic B, Radunovic M, Pucar A, Petrovic SM, Grubisa I, Lazarevic M, Brkic Z, Vukcevic JK, Milasin J, 2018. The presence of periopathogenic bacteria in subgingival and atherosclerotic plaques–An age related comparative analysis. *The Journal of Infection in Developing Countries*. 12(12):1088-95.

Kario K, Matsuo T, Kobayashi H, Hoshide S, Shimada K, 2001. Hyperinsulinemia and hemostatic abnormalities are associated with silent lacunar cerebral infarcts in elderly hypertensive subjects. *Journal of the American College of Cardiology*, 37(3), pp.871–877.

Kebschull M, Demmer RT, Papapanou PN., 2010. "Gum bug, leave my heart alone!"--epidemiologic and mechanistic evidence linking periodontal infections and atherosclerosis. *Journal of Dental Research.* 89(9):879-902.

Kinane DF, Galicia JC, Gorr SU, Stathopoulou PG, Benakanakere M, 2008. *P. gingivalis* interactions with epithelial cells. *Frontiers in Bioscience*. 13:966-84.

Kotsilkov K, Popova C, Boyanova L, Setchanova L, Mitov I, 2015. Comparison of culture method and real-time PCR for detection of putative periodontopathogenic bacteria in deep periodontal pockets. *Biotechnology & Biotechnological Equipment*. 29(5):996-1002.

Kumar PS, Leys EJ, Bryk JM, Martinez FJ, Moeschberger ML, Griffen AL, 2006. Changes in periodontal health status are associated with bacterial community shifts as assessed by quantitative 16S cloning and sequencing. *Journal of clinical microbiology*. 44(10):3665-73.

Lanter BB, Sauer K, Davies DG, 2014. Bacteria present in carotid arterial plaques are found as biofilm deposits which may contribute to enhanced risk of plaque rupture. *MBio*.5(3):e01206-14.

Leong XF, Ng CY, Badiah B, Das S, 2014. Association between hypertension and periodontitis: possible mechanisms. *The Scientific World Journal*. 2014. Article ID 768237

Leys EJ, Griffen AL, Strong SJ, Fuerst PA, 1994. Detection and strain identification of *Actinobacillus actinomycetemcomitans* by nested PCR. *Journal of clinical microbiology*. 32(5):1288-94.

Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME, Taubert KA, Newburger JW, Gornik HL, Gewitz MH, Wilson WR, 2012. Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association? A scientific statement from the American Heart Association. *Circulation*. 125(20):2520-44.

Loesche WJ, Schork A, Terpenning MS, Chen YM, Kerr C, Dominguez BL, 1998. The relationship between dental disease and cerebral vascular accident in elderly United States veterans. *Annals of periodontology*, 3(1), 161-174.

Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ, 2006. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *The Lancet*. 367(9524):1747-57.

Maeda N, Okamoto M, Kondo K, Ishikawa H, Osada R, Tsurumoto A, Fujita, H, 1998. Incidence of *Prevotella intermedia* and *Prevotella nigrescens* in periodontal health and disease. *Microbiology and immunology*, 42(9), 583-589.

Maekawa T, Takahashi N, Honda T, Yonezawa D, Miyashita H, Okui T, Tabeta K, Yamazaki K, 2010. *Porphyromonas gingivalis* antigens and interleukin-6 stimulate the production of monocyte chemoattractant protein-1 via the upregulation of early growth response-1 transcription in human coronary artery endothelial cells. *Journal of vascular research*, 47(4):346-54.

Mahalakshmi K, Krishnan P, Arumugam SB, 2017. "Association of periodontopathic anaerobic bacterial co-occurrence to atherosclerosis"–A cross-sectional study. *Anaerobe*. 44:66-72.

Malhotra S, Parkash H, 2013. Coronary artery disease and periodontitis-A prospective study. *Journal International Medical Sciences Academy*, 26(2), 93-7.

Mattila KJ, Nieminen MS, Valtonen VV, Rasi VP, Kesäniemi YA, Syrjälä SL, Jungell PS, Isoluoma M, Hietaniemi K, Jokinen MJ, 1989. Association between dental health and acute myocardial infarction. *Bmj*, 298(6676):779-81.

Meyer DH, Lippmann JE, Fives-Taylor PM, 1996. Invasion of epithelial cells by *Actinobacillus actinomycetemcomitans*: a dynamic, multistep process. *Infection and immunity*, 64(8), 2988-2997.

Miettinen H, Lehto S, Saikku P, Haffner SM, Rönnemaa T, Pyörälä K, Laakso M, 1996. Association of *Chlamydia pneumoniae* and acute coronary heart disease events in non-insulin dependent diabetic and non-diabetic subjects in Finland. *European heart journal*. May;17(5):682-8.

Milasin J, Jakoba N, Stefanovic D, Sopta J, Pucar A, Lekovic V, Kenney BE. 2011. Periodontal Inflammation as Risk Factor for Pancreatic Diseases. *Inflammatory Diseases-A Modern Perspective*, 12, 444-3.

Milward MR, Chapple IL, Wright HJ, Millard JL, Matthews JB, Cooper PR, 2007. Differential activation of NF-kappaB and gene expression in oral epithelial cells by periodontal pathogens. *Clinical & Experimental Immunology*. May;148(2):307-24.

Moore WE, Moore LV, 1994. The bacteria of periodontal diseases. *Periodontology 2000*. 5:66-77.

Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jiménez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB; American Heart Association Statistics Committee; Stroke Statistics Subcommittee. 2016. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation*. 133(4):e38-360.

Mysak J, Podzimek S, Sommerova P, Lyuya-Mi Y, Bartova J, Janatova T, Prochazkova J, Duskova J, 2014. *Porphyromonas gingivalis*: major periodontopathic pathogen overview. *Journal of Immunology Research*, 2014:476068.

Nadkarni MA, Martin FE, Jacques NA, Hunter N, 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*. 148(Pt 1):257-66.

Naghavi M & GBD 2013 Mortality and Causes of Death Collaborators, 2015. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*, 385(9963):117–71.

Nasry HA1, Preshaw PM, Stacey F, Heasman L, Swan M, Heasman PA, 2006. Smoking cessation advice for patients with chronic periodontitis. *British Dental Journal*.11;200(5):272-5; discussion 265.

Nocker A, Camper AK, 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. *FEMS Microbiology Letters*. 291(2):137-42.

Okoro CA, Balluz LS, Eke PI, Ajani UA, Strine TW, Town M, Mensah GA, Mokdad AH. Tooth loss and heart disease: findings from the Behavioral Risk Factor Surveillance System. *American Journal of Preventive Medicine*. 29(5 Suppl 1):50-6.

Pihlstrom BL, Michalowicz BS, Johnson NW, 2005. Periodontal diseases. *Lancet.* 366(9499):1809-20.

Pizzo G, Guiglia R, Lo Russo L, Campisi G, 2010. Dentistry and internal medicine: From the focal infection theory to the periodontal medicine concept. *European Journal of Internal Medicine*, 21(6):496–502.

Popović M, Smiljanić K, Dobutović B, Syrovets T, Simmet T, Isenović ER., 2012. *Human*

cytomegalovirus infection and atherothrombosis. *Journal of Thrombosis and Thrombolysis*. 33(2):160-72.

Pucar A, Milasin J, Lekovic V, Vukadinovic M, Ristic M, Putnik S, Kenney EB., 2007. Correlation Between Atherosclerosis and Periodontal Putative Pathogenic Bacterial Infections in Coronary and Internal Mammary Arteries. *Journal of Periodontology*, 78(4), pp.677–682.

Rahbar A, Söderberg-Nauclér C, 2005. *Human cytomegalovirus* infection of endothelial cells triggers platelet adhesion and aggregation. *Journal of Virology* 79(4):2211-20

Reddy KS, Yusuf S, 1998. Emerging epidemic of cardiovascular disease in developing countries. *Circulation*, 97(6):596–601.

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS, 2003. Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in Microbiology*. 11(2):94-100.

Riggio MP, Lennon A, Roy KM, 1998. Detection of *Prevotella intermedia* in subgingival plaque of adult periodontitis patients by polymerase chain reaction. *Journal of Periodontal Research*. 33(6):369-76.

Romano F, Barbui A, Aimetti M, 2007. Periodontal pathogens in periodontal pockets and in carotid atheromatous plaques. *Minerva stomatologica.* 56(4):169-79.

Roy F, Vanterpool E, Fletcher HM, 2006. HtrA in *Porphyromonas gingivalis* can regulate growth and gingipain activity under stressful environmental conditions. *Microbiology*. 152(Pt 11):3391-8.

Saito T, Ishihara K, Kato T, Okuda K., 1997. Cloning, expression, and sequencing of a protease gene from *Bacteroides forsythus* ATCC 43037 in *Escherichia coli*. *Infection and Immunity*. 1997 Nov;65(11):4888-91.

Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y., 2001. Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. *Microbiology and Immunology*. 45(1):39-44.

Santos CF, Sakai VT, Machado MA, Schippers DN, Greene AS., 2004. Reverse transcription and polymerase chain reaction: principles and applications in dentistry. *Journal of Applied Oral Science*.12(1):1-11.

Sanz M, Lau L, Herrera D, Morillo JM, Silva A., 2004. Methods of detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *Journal of Clinical Periodontology*. 31(12):1034-47.

Schochetman G, Ou CY, Jones WK, 1988. Polymerase Chain Reaction. *The Journal of Infectious Diseases*.158(6):1154-7.

Seymour GJ, Ford PJ, Cullinan MP, Leishman S, Yamazaki K., 2007. Relationship between periodontal infections and systemic disease. *Clinical Microbiology and Infection*. 13 Suppl 4:3-10.

Seymour GJ, Ford PJ, Cullinan MP, Leishman S, West MJ, Yamazaki K., 2009. Infection or inflammation: the link between periodontal and cardiovascular diseases. *Future Cardiology*. 5(1):5-9.

Sharma A, Inagaki S, Honma K, Sfintescu C, Baker PJ, Evans RT., 2005. *Tannerella forsythia*-induced alveolar bone loss in mice involves leucine-rich-repeat BspA protein. *Journal of Dental Research*. 84(5):462-7.

Sharma A, Sojar HT, Glurich I, Honma K, Kuramitsu HK, Genco RJ., 1998. Cloning, expression, and sequencing of a cell surface antigen containing a leucine-rich repeat motif from *Bacteroides forsythus* ATCC 43037. *Infection and Immunity*. 66(12):5703-10.

Sharma A, 2010. Virulence mechanisms of *Tannerella forsythia*. *Periodontology 2000,* 54(1):106–116.

Sim SJ, Kim HD, Moon JY, Zavras AI, Zdanowicz J, Jang SJ, Jin BH, Bae KH, Paik DI, Douglass CW., 2008. Periodontitis and the risk for non-fatal stroke in Korean adults. *Journal of Periodontology*. 79(9):1652-8.

Smalley JW, 1994. Pathogenic mechanisms in periodontal disease. *Advances in Dental Research*. 8(2):320-8.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent Jr RL, 1998. Microbial complexes in subgingival plaque. *Journal of clinical periodontology.* 25(2):134-44.

Soto-Barreras U, Olvera-Rubio JO, Loyola-Rodriguez JP, Reyes-Macias JF, Martinez-Martinez RE, Patiño-Marin N, Martinez-Castañon GA, Aradillas-Garcia C, Little JW., 2013. Peripheral arterial disease associated with caries and periodontal disease. *Journal of Periodontology*. 84(4):486-94.

Stratagene. Introduction to Quantitative PCR: Methods and Applications Guide.

Stringhini S, Sinon F, Didon J, Gedeon J, Paccaud F, Bovet P., 2012. Declining stroke and myocardial infarction mortality between 1989 and 2010 in a country of the African region. *Stroke*, 43(9), pp.2283–2288.

Szulc M, Kustrzycki W, Janczak D, Michalowska D, Baczynska D, Radwan-Oczko M., 2015. Presence of Periodontopathic Bacteria DNA in Atheromatous Plaques from Coronary and Carotid Arteries. *BioMed Research International*. 2015:825397.

Tanner AC, Izard J, 2006. *Tannerella forsythia*, a periodontal pathogen entering the genomic era. *Periodontology 2000*. 42:88-113.

Thorstensson H, Hugoson A, 1993. Periodontal disease experience in adult long-duration insulin-dependent diabetics. *Journal of Clinical Periodontology*. 20(5):352–8.

Topcuoglu N, Kulekci G, 2015. Anaerobe 16S rRNA based microarray analysis of ten periodontal bacteria in patients with different forms of periodontitis. *Anaerobe*, 35(Pt A):35-40.

Tremolada S, Delbue S, Ferraresso M, Carloni C, Elia F, Larocca S, Bortolani E, Ferrante P, 2011. Search for genomic sequences of microbial agents in atherosclerotic plaques. *International Journal of Immunopathology and Pharmacology*. 24(1):243-6.

Unal B, Critchley JA, Capewell S, 2004. Explaining the Decline in Coronary Heart Disease Mortality in England and Wales between 1981 and 2000. *Circulation*, 109(9):1101–1107.

Valasek MA, Repa JJ, 2005. The power of real-time PCR. *American Journal of Physiology - Advances in Physiology Education*, 29(3):151–9.

Valtonen VV, 1999. Role of infections in atherosclerosis. *American Heart Journal*. 138(5 Pt

2):S431-3.

Virok D, Kis Z, Kari L, Barzo P, Sipka R, Burian K, Nelson DE, Jackel M, Kerenyi T, Bodosi M, Gönczol E, Endresz V., 2006. *Chlamydophila pneumoniae* and *human cytomegalovirus* in atherosclerotic carotid plaques--combined presence and possible interactions. *Acta Microbiologica et Immunologica Hungarica*. 53(1):35-50.

Xie H, Cai S, Lamont RJ., 1997. Environmental regulation of fimbrial gene expression in *Porphyromonas gingivalis*. *Infection and Immunity*. 65(6):2265-71.

Yang NY, Zhang Q, Li JL, Yang SH, Shi Q., 2014. Progression of periodontal inflammation in adolescents is associated with increased number of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, and *Fusobacterium nucleatum*. *International Journal of Paediatric Dentistry*. 24(3):226-33.

Yi L, Lin JY, Gao Y, Feng ZJ, Wang DX, 2008. Detection of *human cytomegalovirus* in the atherosclerotic cerebral arteries in Han population in China. *Acta Virologica*. 2008;52(2):99-106.

Yusuf S, Vaz M, Pais P, 2004. Tackling the challenge of cardiovascular disease burden in developing countries. *American Heart Journal*, 148(1):1–4.

Zu J, Lu H, Gmu R, 2007. Uncultivated *Tannerella* BU045 and BU063 are slim segmented filamentous rods of high prevalence but low abundance in inflammatory disease-associated dental plaques. *Microbiology*. 153(11):3809-16.

BIOGRAPHY

Ibrahim Yousif Kannosh was born on January 1st 1963 in Misurata, Libya. He completed his high school education in Misurata, and enrolled at the Libyan Academy in Tripoli, Department of Natural Sciences (Program Microbiology), where he graduated in 2010 and was awarded the degree of Master of Science. The title of his master thesis was „Spreading of coliform bacteria 0157:H7 responsible for childhood diarrhea in Misurata".

Supported by the Ministry of Education of Libya, Ibrahim Kannosh enrolled in the PhD studies at the University of Belgrade - Faculty of Biology (Program Biology, Module Microbiology) in 2014/15 academic year. His PhD research was in the field of clinical microbiology; more precisely he studied the links between periodontitis and atherosclerosis and myocardial infarction by quantitative and qualitative assessment of periodontal pathogens in patient's mouth and blood vessels or thrombi. He carried out the research at the Laboratory of Molecular Genetics, Department of Human Genetics, School of Dental Medicine, University of Belgrade, under the supervision of Professor Jelena Milašin.

During his PhD studies Ibrahim Yousif Kannosh has published five scientific articles in renowned international journals and has participated at three international congresses.

Образац 5.

Изјава о ауторству

Име и презиме аутора **IBRAHIM YOUSIF KANNOSH**

Број индекса **Б3005/2014**

Изјављујем

да је докторска дисертација под насловом

„Присуство оралних патогених микроорганизама у атерому и тромбу пацијената са атеросклерозом и инфарктом миокарда“ („Presence of oral pathogenic microorganisms in atheroma and thrombus of patients with atherosclerosis and myocardial infarction“)

- резултат сопственог истраживачког рада;
- да дисертација у целини ни у деловима није била предложена за стицање друге дипломе према студијским програмима других високошколских установа;
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио/ла интелектуалну својину других лица.

Потпис аутора

У Београду, 15.12.2019. године

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Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора **IBRAHIM YOUSIF KANNOSH**

Број индекса **Б3005/2014**

Студијски програм **Биологија, микробиологија**

Наслов рада **„Присуство оралних патогених микроорганизама у атерому и тромбу пацијената са атеросклерозом и инфарктом миокарда“ („Presence of oral pathogenic microorganisms in atheroma and thrombus of patients with atherosclerosis and myocardial infarction“)**

Ментор **Др Јелена Кнежевић-Вукчевић, редовни професор, Универзитет у Београду, Биолошки факултет**

Др Јелена Милашин, редовни професор, Универзитет у Београду, Стоматолошки факултет

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, 15.12.2019. године

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Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

„Присуство оралних патогених микроорганизама у атерому и тромбу пацијената са атеросклерозом и инфарктом миокарда“ („Presence of oral pathogenic microorganisms in atheroma and thrombus of patients with atherosclerosis and myocardial infarction“)

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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