

## **Bacterial colonization patterns of periodontal pockets in different ages**

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**Key words** Periodontitis, Bacterial infection, Real-Time PCR, Red complex bacteria percentage

**Summary** The aim of this study was to investigate subgingival bacterial composition of untreated Italian subjects with aggressive and chronic periodontitis.

The total bacterial load, pathogenic bacteria belonging to "red" and "orange" complexes and *Aggregatibacter actinomycetemcomitans* were determined by Real-Time PCR in 1216 patients. Data were analysed by looking for relationships between bacteriological parameters, age and periodontal probing depth.

The obtained results showed a significant higher number of red complex bacteria in older rather than in younger patients. The total number of bacteria and the presence of *A. actinomycetemcomitans* did not clearly associate with an age group.

### Profili di colonizzazione batterica delle tasche parodontali in varie classi di età

**Parole chiave** Parodontite, Colonizzazione batterica, Real-Time PCR, Percentuale dei batteri del complesso rosso

**Riassunto** Lo scopo di questo studio è stato quello di caratterizzare la flora batterica subgingivale di un campione di 1216 pazienti italiani affetti da parodontite cronica e aggressiva mai sottoposti ad alcun trattamento parodontale.

L'analisi, eseguita con metodica Real-Time PCR su campioni di placca, ha fornito la quantificazione della carica batterica totale e dei principali batteri responsabili dell'insorgenza e della progressione della malattia parodontale quali *Aggregatibacter actinomycetemcomitans* e i patogeni appartenenti ai complessi definiti da Socransky "rosso" e "arancione". I dati ottenuti sono stati analizzati per rilevare eventuali correlazioni tra i parametri biologici, età e profondità delle tasche parodontali.

I risultati ottenuti hanno evidenziato che la percentuale dei batteri appartenenti al complesso rosso aumenta con l'aumentare dell'età dei pazienti. La carica batterica totale e la presenza di *A. Actinomycetemcomitans* non risultano invece associate con l'età.

## Introduction

Periodontitis is ubiquitous throughout the world's and is among the most prevalent and costly health problem affecting industrialised societies. The impact of periodontal disease on individuals and communities as a result of the pain and suffering, impairment of function and reduced quality of life they cause, is considerable. The epidemiological data in the literature are few and controversial. The World Health Organization estimates that severe periodontitis occurs in 5-20% of adults worldwide, while milder forms of disease occurs in approximately 35-50% of the adult population (Petersen et al 2005). There are recent epidemiological studies conducted in industrialized countries that assess the prevalence of periodontal disease. A 30-year study of periodontal conditions in Sweden, showed a significant improving of oral hygiene and periodontal health but interestingly the proportion of Swedish population that suffering of advanced periodontitis remained the same (Hugoson et al. 2008). A study in Norway monitoring the periodontal health of 35 years-old in Oslo from 1973 to 2003 showed improvements in oral hygiene and decreasing of the prevalence of periodontitis over the study period (Skudutyte-Rysstad et al. 2007). In the USA the prevalence of periodontal disease showed a decrease for all racial group from 1988 to 2000, but the burden of periodontal disease remain on the disadvantages and poor population groups (Borrell et al. 2005). A review written by Hugoson et al. for the Sixth European Workshop on Periodontology evaluates the global trends in the change in prevalence of periodontitis over the last 30 years. The data indicate a possible trend of a lower prevalence of periodontitis in recent years in Europe and USA but the reported change is mainly in gingivitis and mild-to-moderate periodontal disease (Hugoson et al. 2008). According to data published by the Italian Society of Periodontal Disease (SIDP) in 2003, 60% of adults in Italy suffer from various degrees of periodontal disease, 10-14% of which are serious and advanced. There is a drastic increase in the incidence of periodontitis in the 35-44 age range ([www.sidp.it](http://www.sidp.it)). According to our experience the SIDP data are more reliable than those listed above that are probably underestimated.

A position paper prepared by the Research, Science and Therapy Committee of the American Academy of Periodontology in 2005 represents the position of the Academy in regard to the current state of knowledge about the epidemiology of

periodontal disease and it replaces the version published in 1996. The paper states that the more important determinants of periodontitis are:

- Age
- Gender: periodontitis is generally more prevalent in males than in females.
- Socioeconomic Status and racial/ethnic differences
- Individual genetics profile related to key regulator of the host inflammatory. Evidence suggests that there is a significant genetic component to susceptibility and resistance to periodontal disease. (Zhang et al. 2011)
- Smoke: the risk of periodontitis attributable to tobacco, compared to its non-use, is in the order of 2.5 to 6.0 or even higher (Bergstrom and Preber 1994; Academy Report 2005).
- Putative periodontopathogens in subgingival plaque

Periodontal disease is a polymicrobial infection of the periodontal tissues caused by pathogenic bacteria and characterised by a more or less progressive destruction of the periodontal ligament and alveolar bone, which may result in tooth loss. According to the latest scientific evidence, periodontal disease is also a risk factor for the development of important systemic pathologies, such as respiratory illness (Wang et al. 2009) cardiovascular problems (Nakano et al. 2009), diabetes (Dunning T. 2009) and pregnancy complications (Katz et al. 2009). The transition from a “healthy” to a “disease-associated” subgingival microbiota can be affected by a number of factors including pH, oxygen levels, temperature, osmotic pressure and oxidation–reduction potential (Socransky & Haffajee 2005). The risk of disease occurrence has also been shown to be variously associated both genetic, microbiological and environmental factors (Stabholz et al. 2010). Periodontitis is traditionally classified as chronic (CP) or aggressive (AP) in relation to some clinical parameters such as the age of onset or detection, rate of progression, patterns of destruction, signs of inflammations and amount of plaque and calculus (1). However, clinical distinction between chronic and aggressive periodontitis is still not clear cut (Armitage & Cullinan 2010).

In microbiological terms periodontal disease occurs when there is a destruction in the host-microbe homeostasis associated with health (Armitage 2010). In particular a form of aberrant inflammation is developed resulting from a shift in the microbial communities of the gingival sulcus from predominantly aerobic, gram-positive, commensal, oral bacterial species to predominantly gram-negative,

anaerobic, chemorganotrophic, and proteolytic strains organised in a biofilm (Slots & Taubman 1992). The dental plaque presence of members of bacterial species that constitute the “red complex”, such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, have previously been associated with advanced periodontitis and perimplantitis, while the presence of bacteria of the “orange complex”, such as strains of the species *Fusobacterium nucleatum* ssp. *polymorphum* and *Prevotella intermedia*, has been associated with either initial and moderate forms of periodontitis or with the recovery phase (Socransky et al. 1998). Bacterial species belonging to the orange complex have been found to precede colonization by species of the red complex (Socransky et al. 1998). Moreover, the increased prevalence, proportion and absolute numbers of bacteria in deep periodontal pockets when compared to moderate or shallow pockets have been reported for species of both complexes (Gmur et al. 1989, Socransky et al. 1991, 1998, Simonson et al. 1992, Kojima et al. 1993, Wolff et al. 1993, Ali et al. 1994, Pederson et al. 1994, Kamma et al. 1995, Kigure et al. 1995, Haffajee et al. 1998; Socransky et al. 2000). More recently, strains of the species *Aggregatibacter* (ex *Actinobacillus*) *actinomycetemcomitans* have been claimed to be among the most important etiological microorganisms involved in aggressive forms of periodontitis (Heubek et al. 2004) because of their ability to produce a powerful leukotoxin (Haraszthy et al. 2000). However, the microbiota found in periodontal infections (both chronic and aggressive forms) shares a large number of taxa and no evidences have been provided for tight association of a bacterial taxon with periodontal disease, hampering the application of the classical one-pathogen/one disease model (Armitage 2010).

Microbiological tests, which provide the quantization of the more important periodontopathogens, are fundamental tools for the clinician in both the pre-operative and post-operative phases. These tools provide an accurate description of the microbial component of subgingival plaque that is essential to develop a targeted therapy and to verify the efficiency of treatments. With the routine use of molecular biology techniques, commercial tests based on 16S rRNA gene Real-Time polymerase chain reaction (Real-Time PCR, or qPCR) offer high sensitivity and the possibility of species-specific detection. Consequently, qPCR tests providing quantitative and qualitative analysis for the major etiologic agents of periodontitis (Kuboniwa et al. 2004, Sanz et al. 2004) are the only useful chairside tests for

developing more specific treatments for periodontal diseases. The Real-Time PCR technique allows for the determination of the percentage of each bacterial species using specific primers, while the total bacterial load is estimated using universal bacterial primers for the 16S rRNA gene (Sanz et al. 2004). This assay demonstrated a high degree of specificity and reproducibility in quantifying pathogenic species. Furthermore, Real-time PCR also has high sensitivity for detecting numerous bacterial species even if present in very low numbers in the subgingival plaque. Using this approach, several investigators detected and estimated the percentage of bacteria of red and orange complexes in subgingival plaques (Kuboniwa et al. 2004, Suzuki et al. 2005, Boutaga et al. 2006).

Previous studies have shown that distribution of periodontal pathogens can be related to geographic locations, race and ethnicity (Haffajee et al. 2004, 2005, Lopez et al. 2004, Ximenez-Fyvie et al. 2006). Very few studies have investigated periodontal microbiota in Italian populations (König et al. 2005, Kim et al. 2009). Very recently, Armitage (Armitage 2010), pointed out the need for studies based on culture-independent microbiological techniques to increase the knowledge about the microbiological features of periodontitis and their relationships with environmental and clinical variables. In this respect, to our knowledge, no reports on possible correlations between the presence and percentage of periodontopathogenic bacteria with patient age have been performed.

The aim of this study was to assess the correlation between the variation in subgingival bacterial composition (determined by qPCR) of untreated Italian subjects and patients' age.

## **Materials and Methods**

### **Subject population**

Samples of 1216 Italian untreated subjects from IRF in Microdentistry (Florence) were analysed. The demographic and clinical characteristics of the population are presented in Table 1 and Supplemental Material S1. Informed consent was obtained from all subjects.

### **Inclusion criteria**

Diagnosis of disease was made on the basis of dental clinical parameters, including periodontal probing depth (PPD), bleeding on probing (BOP), suppuration

**Table 1 - Demographic characteristics of study subjects. Mean value of age and PPD ( $\pm$ SD) and the percentage of females and smokers of 1216 caucasian subjects (n=1216)**

Variable	Mean
Age (mean $\pm$ SD)	50.1 $\pm$ 12.1
Females (percentage)	730 (60.03%)
Smokers (percentage)	283 (23.20%)
Ethnicity (percentage)Caucasian	1216 (100%)
PPD (mm) (mean $\pm$ SD)	5.6 $\pm$ 2.9
*SD, standard deviation. PPD, probe pocket depth	

(PUS), and radiographic patterns of alveolar bone destruction (Armitage 1999; Wiebe et al. 2000). Only patients having all PPD higher than 3 mm were included in the study. Clinical evaluation of PPD was performed using Florida Probe

(www.florida probe. com) a computerized periodontal probing that allows tests comparable over time and independent from the operator.

### Exclusion criteria

Exclusion criteria included known systemic diseases, history and/or the presence of other infections, systemic antibiotic treatment in the preceding three months and pregnancy or lactation in females.

### Microbiological examination

#### Sample Collection

Sampling was carried out following the procedures reported in the BPA kit (Bacterial Periodontal Assessment, Biomolecular Diagnostics, Italy) after drying the area and removing supragingival plaque. Subgingival plaque samples were collected with sterile paper points inserted for one minute into the deepest pockets (choosing at least one pocket for each quadrant) and stored at 4°C in a sterile tube. Five samples per patient were taken from different sites having PPD $\geq$ 3 mm and pooled together.

#### Assessment of periodontopathic bacteria

Plaque samples were sent to Biomolecular Diagnostics (Firenze, Italy). The DNA extraction was performed using QIAextractor (QIAGEN Inc., GmbH, Hilden, Germany) according to the manufacturer's protocol. Real-time PCR with SYBR-green assays were carried out using a Rotor Gene 3000 (Corbett) apparatus. For amplification reactions, duplicate samples were routinely used. About 40 ng of

DNA eluted was used for bacterial detection of the most important periodontopathogens, including *P. gingivalis*, *T. denticola*, and *T. forsythia*, *E. nucleatum* ssp. *polymorphum*, *P. intermedia* and *A. Actinomycetemcomitans* using species-specific primers for the 16S rRNA gene. Bacterial titres are expressed as number of cells per plaque sample. The total number of bacterial cells was also determined using a universal primers set. The standard curve was analyzed for each evaluated bacterium and using the universal primers set against a serial dilution of each bacterial DNA corresponding to  $10^2$ – $10^7$  cells. The negative control was a Real Time PCR mix without DNA.

### Statistical analysis

Statistical analysis was carried out with the software STATISTICA 7.0 (Statsoft, Inc., Tulsa, OK, USA). Factor analysis was used to evaluate the relative contribution of each variable of the dataset. The Kruskal-Wallis test with Bonferroni contrast was then performed to estimate the significance of reported differences. Spearman correlation was used to investigate the relationships between clinical variables and the presence of bacteria.

## Results

### Clinical data

The population recruited for our study is only composed of Italian individuals. The mean age of the participants is 50.1 years. There is a prevalence of females (60.03%) and non-smokers (76.8%) with a mean PPD value of 5.6 mm (Table 1).

### Microbiological data

The mean total number of bacteria in the dental plaque samples was  $2.09 \times 10^7$  cells/plaque sample (Table 2). Non-smokers showed higher mean values than smokers ( $P < 0.03$ ) and females had slightly more bacteria than males ( $P < 0.7$ , not significant). The percentage of pathogenic bacteria was 16.5% for the whole dataset, with females having a mean value of 15.7% while males had a mean value of 16.4% ( $P < 0.7$ , not significant). Non-smokers had slightly more pathogens than smokers ( $P < 0.5$ , not significant).

In order to quantitatively evaluate which parameter(s) considered in our analysis (age, PPD, total bacteria present and percentages of different bacterial groups)

Table 2 - Total number of bacteria (TOT BAC) and percentage of pathogenic bacteria (% PTGS) in the sampled dataset\*

	Tot BAC $\times 10^6$ cells/plaque sample	(% PTGS)
All dataset	20.9 $\pm$ 1.60	16.5 $\pm$ 0.55
Males	19.4 $\pm$ 2.20	16.4 $\pm$ 0.96
Females	21.87 $\pm$ 2.1	15.7 $\pm$ 0.71
Smokers	13.9 $\pm$ 1.5	13.9 $\pm$ 1.04
Nonsmokers	23.0 $\pm$ 2.04	16.86 $\pm$ 0.66
<b>Age classes:</b>		
$\leq 20$ y	21.8 $\pm$ 10.80	6.4 $\pm$ 1.88
21-30	15.9 $\pm$ 5.79	7.6 $\pm$ 1.32
31-40	23.9 $\pm$ 6.30	11.4 $\pm$ 1.43
41-50	25.3 $\pm$ 3.19	16.1 $\pm$ 0.98
51-60	18.2 $\pm$ 1.86	16.5 $\pm$ 0.87
61-70	17.4 $\pm$ 3.80	15.4 $\pm$ 1.45
71-80	11.0 $\pm$ 1.54	21.3 $\pm$ 3.24
81-90	8.17 $\pm$ 1.28	13.65 $\pm$ 6.63

\*The means of total number of bacteria and the mean percentage of pathogens estimated by Real-Time PCR (see Materials and Methods) in the whole dataset, and related to gender, smoke and age classes of patients;  $\pm$ , standard error.

most affected the diversity of our dataset, a factor analysis was carried out. Results are reported in Table 3. The first two factors, which explained 47.12% of the total dataset variance, the percentage of pathogenic bacteria and of bacteria belonging to the red complex, were the highest contributors to the first factor. The second factor was mostly determined by the age of patients. A graphical representation of the respective weights of variables is shown as the principal component analysis (Figure 1). The percentage of pathogenic bacteria (% PTGS) and the red complex bacteria (% RED) were clustered together forming a different group with respect to the percentage of orange complex bacteria (% ORG), PPD, and age. Total bacterial counts (TOT BAC) and the percentages of *A. actinomycetemcomitans* (% A.a.) are the most distantly related to % PTGS and % RED. In the second factor, age was found to be the main discriminator of the dataset.

To better evaluate the relationships between the age of patients and the quantity and quality (presence of bacteria from different complexes) of bacterial flora,

**Table 3 - Factor analysis of the dataset\***

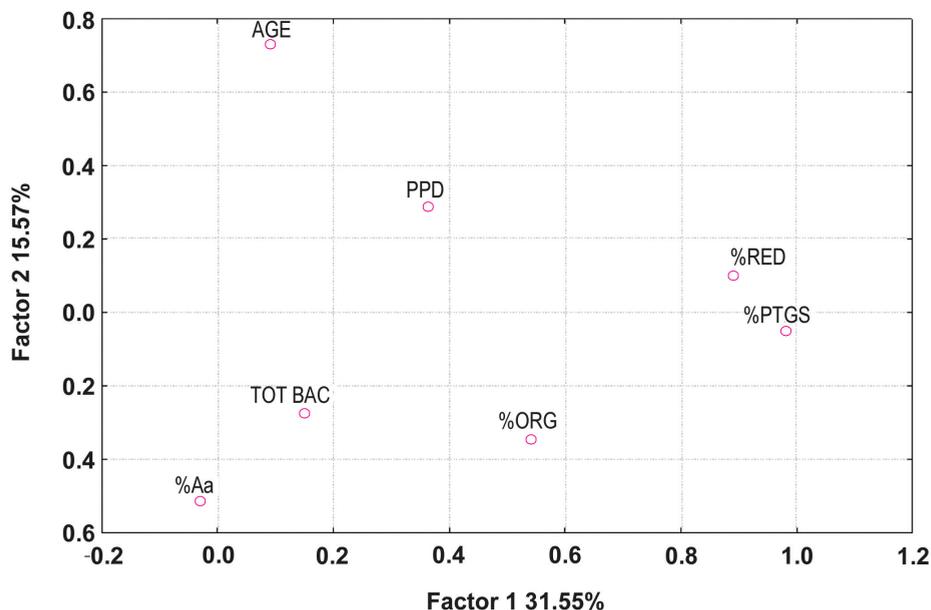
	<b>Factor 1</b>	<b>Factor 2</b>
AGE	0.089850	<b>0.73221</b>
PPD	0.364752	0.288355
Tot BAC	0.144819	-0.264346
%PTGS	<b>0.982249</b>	-0.051339
%A.a.	-0.030088	-0.516395
%RED	<b>0.888459</b>	0.101892
%ORG	0.540053	-0.348600
Explained variance	2.208825	1.090356
Proportion over total	0.315546	0.1155765

\*Factorial weights are shown for each component . Factor analysis is performed including age, PPD, total bacteria present and the percentage of bacteria belonging to different groups (codes as reported in legend of figure 1). The explained variance and the proportion over total variance are also reported. In bold are weights >0.70000.

the dataset was divided into eight classes as reported in Table 2. Total bacterial numbers varied from  $8.17 \times 10^6$  to  $25.3 \times 10^6$  cells/plaque sample in the oldest class (81-90 years old) and in the 41-50 years class, respectively. Pathogens' percentages were highest in the 71-80 years class and lowest in the  $\leq 20$  years class. Then, for each class, the mean percentages of bacteria belonging to the red and orange complexes and of *A. actinomycetemcomitans* were computed (Fig. 2). Bonferroni contrast after a Kruskal-Wallis

test was then used to evaluate the statistical significance of the differences in observed means. Interestingly, the percentages of red complex (Fig. 2A) bacteria was significantly lower in patients 20-40 years old and those older than 50 years. Similarly, the percentages of orange complex bacteria (Fig. 2B) were significantly different (lower) in 20-40 year old patients compared with those who were 40-60 years old. However, in this case, there was not a strict differentiation between a younger and an older group of classes, as the 60-70 year old class and the 70-90 year old class had lower values than the 40-60 year old class. For *A. actinomycetemcomitans* (Fig. 2C), no clear differences were identified in relation to age classes. However, in our dataset, individuals younger than 20, and in the 31-40, 61-70, and 81-90 year old sets, showed slightly higher percentages of *A. actinomycetemcomitans* than the other classes. Overall, the  $\chi^2$  statistic for Kruskal-Wallis was 46.92 ( $P < 0.0001$ ) for orange complex bacteria and 39.65 ( $P < 0.0001$ ) for red complex bacteria. In general, most of the differences were found in the comparisons between younger (less than 40 years old) and older classes (more than 41 years old). To verify that patients younger than 30-40 years old had different microbiological parameters than older patients, two groups of patients were compared, with 36 years as the discriminator between aggressive and chronic

Figure 1 - Principal component analysis of factorial weights



The first two principal components with indicated the percentage of explained variance are shown. % PTGS, percentage of pathogens; % RED, percentage of red complex bacteria; % ORG, percentage of orange complex bacteria; % A.a, percentage of *A. actinomycetemcomitans*, PPD, probing pocket depth; TOT BAC, total number of bacteria; AGE, age of patient.

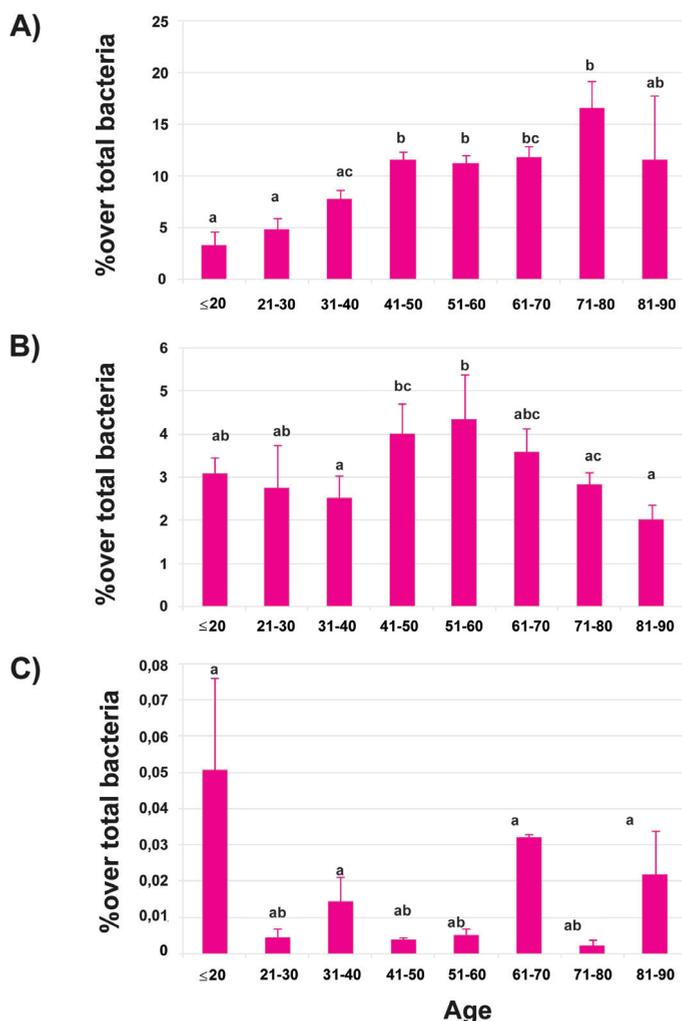
periodontitis. Results from this analysis are shown in Table 4. The percentage of pathogens (% PTGS), red complex bacteria (% RED) and probing pocket depth (PPD) showed significant differences between the two groups.

Spearman correlation analyses were then carried out between PPD and the number and type of bacteria (Table 5). The highest and the very small values for correlation were found between the percentage of red complex bacteria and PPD. The total number of bacteria and the percentage of pathogenic bacteria were also correlated, though at lower values. No correlation was found between PPD and percentages of *A. actinomycetemcomitans* and orange complex bacteria.

## Discussion

Periodontal disease is the result of an altered equilibrium between prevalent periodontal pathogens (i.e., gram-negative anaerobes) and host-compatible species

Figure 2 - Percentage of different bacteria in the different age classes



The percentage of bacteria belonging to the red complex (A), orange complex (B) and the species *A. actinomycetemcomitans* (C) is reported for each age class. Error bars, standard error. Different letters above columns indicate statistically significant ( $P < 0.05$ ) differences after Bonferroni contrast in a Kruskal-Wallis test.

in the oral cavity (Socransky et al. 1998, Van Winkelhoff et al. 2002, Nishihara & Koseki 2004, Byrne et al. 2009). Numerous reports have demonstrated an association between periodontitis and a small subset of microbial species, which includes the

Table 4 - Statistical analysis of differences in patients in relation to two age groups\*

	Kruskal-Wallis' statistics	P-value	Bonferroni contrast
Total bacteria	0.70	0.4 (n.s.)	-27.16
% PTGS	32.28	<0.0001	-183.51
% RED	25.98	<0.0001	-113.80
% ORG	12.99	0.0003	-80,50
% A.a.	0.03	0.8 (n.s.)	2.74
PPD	17.08	<0.0001	-132.27
PUS	1.65	0.2 (n.s.)	-28.91

\* two groups with patients having less than 36 years and more than 37 years were compared for total amount of bacteria, percentage of pathogenic bacteria (PTGS), bacteria of the red (RED) and orange (ORG) complex, PPD and for the presence of pus on probing (PUS). The Kruskal-Wallis' statistics, P-value and Bonferroni contrast are shown. n.s., not significant.

Table 5 - Spearman correlation analysis between PPD and bacteria\*

	rs statistic	P value
Total bacteria	0.09	<0.003
% PTGS	0.21	<0.0001
% RED	0.23	<0.0001
% ORG	0.06	<0.3 (n.s.)
% A.a.	-0.03	<0.4 (n.s.)

\*The rs statistic and p-values are reported. Codes in columns as in Tab. 4.

bacteria of our study, such as *P. gingivalis*, *T. forsythia*, *F. nucleatum* ssp. *polymorphum*, *A. actinomycetemcomitans*, *T. denticola*, and *P. intermedia* (Armitage 2010). A scheme of microbial succession during colonization of the oral ecosystem has been proposed (Socransky et al.

1998, Socransky & Haffajee 2005), according to which after the initial colonization of orange complex bacteria (*F. nucleatum* and *P. intermedia*), the red complex bacteria (*P. gingivalis*, *T. denticola*, and *T. forsythia*) become more dominant, leading to the development of more advanced forms of disease (PPD 6 mm or deeper). *F. nucleatum* ssp. *polymorphum*, the main bacterial species of the orange complex, binds to epithelial cells (Han et al. 2000, Edwards et al. 2006) and has been described as an initiator organism in plaque biofilm development because it co-aggregates with both early and late colonisers (Kolenbrander & London 1993, Shanitzki et al. 1997, Zilm et al. 2007). Some bacteria as *P. gingivalis*, *T. forsythia* and *T. denticola* (red complex bacteria) were frequently found together in periodontal lesions, particularly in sites with deep pockets or more advanced periodontitis (Umeda et

al. 1996, Socransky et al. 1998). In particular *P. gingivalis* is considered to be a major pathogen in human periodontitis and is implicated in certain systemic conditions, such as cardiovascular disease, metabolic disease (diabetes), rheumatoid arthritis (Gaetti-Jardim et al. 2009, Nakano et al. 2009, Wang et al. 2009). *A. actinomycetemcomitans* is a non-motile, gram-negative, capnophilic bacterium that has been strongly implicated in localized and generalized aggressive periodontitis (Darveau et al. 1997, Slots 1999, Faveri et al. 2009) and plays an important role in periodontal destruction (Yang et al. 2004, Orrù et al. 2006). *A. actinomycetemcomitans* has been isolated in a small percentage (among 10-20%) of individuals with periodontitis in Asian, Eurasian and South-American populations (Kim et al. 2009, Cosgarea et al. 2010, Roman-Torres et al. 2010). In agreement with these studies, we observed that 19.9% of our patients showed the presence of *A. actinomycetemcomitans*.

Interestingly, Armitage recently affirmed that despite a great numbers of studies, our knowledge of the comparative microbiology of chronic and aggressive forms of periodontitis are still incomplete (Armitage 2010). At this purpose, our findings about the composition of subgingival plaque revealed that there is a significant difference in the proportion of red complex bacteria with regard to age class. In particular, the analysis of the microbial composition of 1216 patients, divided by classes ages of ten years, demonstrated an increase in the percentage of red complex bacteria with increasing age. Nevertheless, the trends of orange complex bacteria and *A. actinomycetemcomitans* did not show differences with regard to patient age. Furthermore, by setting a discriminator at 36 years of age, that is commonly considered as the age limit for aggressive periodontitis, our results show that the percentages of pathogenic bacteria and of red complex bacteria were significantly differentiated and patient younger than 36 years present a lower percentage of red complex bacteria than patients older than 36 years. Interestingly, there were not relevant clinical differences in PPD between patients with less and more than 36 years (means  $4.7 \pm 2.5$  and  $5.7 \pm 3.0$  mm, respectively, data not shown. This fact suggests that, irrespective from PPD, the percentage of pathogenic, and especially of red complex bacteria, may be useful in discriminating between aggressive and chronic periodontitis, particularly in the range 36-50 years, in which clinical distinction between these two forms is still dubious (Armitage & Cullinan 2010).

The lack of correlation between age and percentage of orange complex bacteria

is in agreement with Socransky's hypothesis of the important role of orange complex bacteria in the beginning of periodontal disease but not as a factor related to the development of periodontitis. Overall, our findings may suggest in agreement with previous reports, that red complex bacteria are the major etiologic agents in periodontitis (Feng & Weinberg 2006). Red complex bacteria have also recently been related to increasing levels of gingival crevicular fluid biomarkers, such as interleukin-1 (IL-1), which have been associated with the immunopathology of periodontitis as a critical determinant of tissue destruction and bone resorption (Teles et al. 2010).

The Socransky model also suggests a relationship between some clinical parameters, such as PPD and the composition of subgingival microbiota. According to other studies, deeper pockets (PPD $\geq$ 6mm), associated with severe periodontitis, showed a greater percentage of red complex species compared to shallow pockets (PPD $\leq$ 4mm) (Socransky & Haffajee 2005, Savage 2009). We found a positive, though very faint, correlation between red complex bacteria and PPD, suggesting that deeper pockets tend to contain a higher proportion of red complex bacteria. Interestingly, the total number of bacteria did not correlate with pathogenic bacteria (except for a clustering near *A. actinomycetemcomitans* (see Fig. 1) and PPD, suggesting that the simple bacterial flora count is not a good indicator of the severity of periodontitis.

It is interesting to note that a new approach, termed "Infectogenomics" (Nibali et al. 2009), has been proposed in the literature to study the correlation between the genetic profile and the host immune response against pathogens in different diseases (such as periodontitis and Crohn's disease). Infectogenomics data show the importance of the clinical use of microbiological chair-side tests based on qPCR, in addition to genetic assessments, to rapidly and economically evaluate the presence and percentages of *P. gingivalis*, *P. intermedia* and *T. forsythia* in relation to total bacterial load, as a fundamental step to define a correct diagnosis and an accurate therapy.

According to this theory, we can explain our microbiological data that in younger patients (<36 years) revealed a lower percentage of red complex bacteria, assuming that in these cases a shorter exposure to periodontal pathogens leads to the development of periodontitis in the presence of a high genetic predisposition. Therefore, we can also speculate that the increased severity of periodontitis in

non-genetically predisposed older age patients is due to a cumulative effect of prolonged exposure to increasing percentages of red complex bacteria with: i) the aging of the immune system and consequent inability to control infections in old age, which is partially due to inefficient communication between macrophages and the tissues. The resulting inability of these phagocytes to control microbial overgrowth in periodontal tissues could lead to chronic persistence of the pathogens and unresolved destructive inflammation (Franceschi et al. 2000), ii) the difficulties of careful oral hygiene, common in the elderly, iii) the decreased efficiency of the mechanisms of washout with age.

To summarize, we have confirmed that infection caused by red complex bacteria is strongly associated with severe forms of periodontitis (deep pockets) and with periodontal disease in older (>36 years) patients. Early detection of these pathogens using microbiological tests could be essential to prevent disease progression through an adequate protocol of follow-up and the percentage of red complex bacteria could be a new approach to discriminate between aggressive and chronic forms of periodontitis, especially in the range 36-50 years.

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