Ageing with HIV - a periodontal perspective

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INTRODUCTION

Symbiosis of more than 700 different bacterial species is important for maintaining the oral cavity homeostasis (As et al., 2005). Physiological roles of the normally present microbiome are to protect the mouth from invasion of pathogenic microorganisms (Ribet and Cossart, 2015) and to provide metabolites and immune modulators necessary for proper development and functioning of the orofacial system (Ruby and Barbeau, 2002). The oral microbiome is continuously changing along with the ageing process (Zapata and Quagliarello, 2015). These changes are finely synchronized with age-related modifications of developing oral tissues, and with the immune system of the oral cavity, both naive and specific. Alterations of immune response caused by human immunodeficiency virus (HIV) infection usually disturb this balance. Progressive decline in host defense due to age and HIV-related changes makes oral tissues more susceptible to the detrimental effects of various pathogens, as well as to side-effects of antiretroviral therapy.

Complex interactions of immune ageing and age-related alterations of oral structures are possible modulators of mouth bacterial load. Although many studies have assessed oral microbial flora in HIV-infected patients, none of them have focused on bacterial composition shifts along with the ageing process in this population.

The aim of the present study was to investigate temporal changes in oral microflora patterns within two different age groups of HIV-infected individuals on highly active antiretroviral therapy (HAART) and to compare them to bacterial composition changes in non-infected individuals.

MATERIALS AND METHODS

Subjects

This study enrolled 60 HIV-infected male patients, 30 younger (≤35 years) and 30 older (≥50 years) treated at the “Dr Kosta Todorovic” Clinic for Infectious and Tropical Diseases, School of Medicine, University of Belgrade. The average HAART treatment duration was approximately 20 months in the younger population and 114 months in the older population. Basic clinical data related to HIV infection are listed in Table 1. Sixty consecutive HIV+ male patients of corresponding age groups (30 younger, 30 older), undergoing regular dental check-up at the Clinics of the School of Dental Medicine, University of Belgrade were recruited as controls. In HIV-infected patients, the mean age in the younger group was 29.33±4.20 (mean ±SD) years, and in the older 59.03±8.17 years. In non-infected patients, the mean age in the younger group was 29.47±3.47 years, whereas in the older group, the mean
age was 61.43±7.50 years. Subjects who were younger than 18 years, HBV or HCV positive, who received antibiotics, anti-inflammatory and immunomodulatory drugs, who had gone through radiotherapy, consumed narcotics, alcohol or oral antiseptics, with a history of any systemic disease, malignancy or oral problem besides caries lesions and periodontal disease, and patients with less than 20 teeth were excluded from the study. There were no statistically significant differences in alcohol and tobacco consumption between HIV+ and HIV- individuals or between younger and older participants. Informed consent was obtained from all participants. All experiments were performed in accordance with the Edinburgh Revision of the Helsinki Declaration, and the study was approved by the Ethical Committees of the School of Medicine and the School of Dental Medicine, University of Belgrade.

Sample collection and DNA extraction
Buccal swab samples were taken from each patient by gently rubbing the inner sides of both buccal regions. Cotton swabs were then separated from the stick with scissors and transferred into 1.5 ml sterile microcentrifuge tubes and stored at -20°C until centrifugation, upper aqueous phase was transferred into new sterile microcentrifuge tube and stored at -20°C until further analyses.

Polymerase chain reaction
Seven periopathogenic bacteria: Aggregatibacter actinomycetemcomitans (Aa), Eikenella corrodens (Ec), Peptostreptococcus micros (Pm), Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Tannerella forsythia (Tf) and Treponema denticola (Td) were detected by polymerase chain reaction (PCR). Five microliters of each sample were added to 20 µl of pre-mixed water PCR solution containing 2.5 µl of 10X DreamTaq Green buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of species specific primers and 1 U of DreamTaq DNA polymerase (Thermo Fisher Scientific™; Waltham, MA, USA). PCR was performed in a thermal cycler (Peqlab PeqSTAR 2X™; Erlangen, Germany) under the following conditions: initial denaturation at 94°C for 3 minutes; 35 runs of denaturation at 94°C for 45 seconds, annealing at appropriate temperature for each pair of primers for 1 minute, and elongation at 72°C for 1 minute; and final elongation at 72°C for 5 minutes. DNA amplicons were separated by electrophoresis on polyacrylamide gels. After that gels were stained in 1X SYBR Safe DNA Gel Stain solution (Life technologies™, Grand Island, NY, USA) for 10 minutes and species specific bands were visualized by exposure to UV light on a transiluminator (Vilber Lourmat TCX-15 MX; Eberhardzell, Germany). All primer sequences and annealing temperatures are listed in Table 2.

Clinical measurements
Clinical examination included intraoral soft tissue inspection and measurement of periodontal parameters at six sites on each tooth with the UNC-15 periodontal probe (Integra Lifesciences® Plainsboro, NJ, USA) by a calibrated examiner. Periodontal tissue status evaluation was based on monitoring the following parameters: plaque index (PI), probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP). The presence of any HIV-related oral condition (HIV-associated gingivitis) was not observed in any of the participants. Further analyses.

Table 1 - Clinical parameters of HIV-infected patients.

<table>
<thead>
<tr>
<th>Species</th>
<th>Age (years)</th>
<th>CD4 count (cells/µl)</th>
<th>pVL (copies × 10³/ml)</th>
<th>Presence of infection (months)</th>
<th>Therapy duration (months)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Younger</td>
<td>29.3 ± 0.5</td>
<td>420.3 ± 212.7 (66-973)</td>
<td>73.1 ± 127.1 (0.0-403.0)</td>
<td>27.6 ± 26.9 (1.1-110.7)</td>
<td>20.2 ± 18.0 (0.8-74.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Older</td>
<td>59.0 ± 8.2</td>
<td>514.7 ± 328.3 (116-1368)</td>
<td>52.3 ± 93.2 (0.0-368.0)</td>
<td>117.0 ± 68.5 (11.8-204.0)</td>
<td>114.3 ± 68.9 (7.5-204.0)</td>
<td>0.546</td>
</tr>
</tbody>
</table>

Table 2 - List of primers used for PCR, their annealing temperatures (Ta) and product sizes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence of primers (5’ → 3’)</th>
<th>Ta (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregatibacter actinomycetemcomitans</td>
<td>Forward: GCTAATACCCGGTGAGATCGG  Reverse: ATTTCACACCTCACTTAAAGGT</td>
<td>55</td>
<td>500</td>
</tr>
<tr>
<td>Eikenella corrodens</td>
<td>Forward: CTAATACCCGATCATCTAGCAATCGG  Reverse: CTAACGAGAATCAGGTTGCCC</td>
<td>55</td>
<td>688</td>
</tr>
<tr>
<td>Peptostreptococcus micros</td>
<td>Forward: AGAGGGTGATCGTGCTGACG  Reverse: ATATCATGCGATTCTGTGGCTC</td>
<td>55</td>
<td>207</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>Forward: AGCAGCTTGCCGCACTCTGCAG  Reverse: ATGTTAGCAGTACCTTCTGATGT</td>
<td>55</td>
<td>400</td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>Forward: CGGTGACAAAAAGATTACTCAGGTGTAAGA  Reverse: CGCGTTTAAATCCCCCAACAA</td>
<td>55</td>
<td>259</td>
</tr>
<tr>
<td>Tannerella forsythia</td>
<td>Forward: GCGTATGCTACCTGGGCAGCA  Reverse: TGCTTCAAGTGTCAGTATGCTCT</td>
<td>55</td>
<td>600</td>
</tr>
<tr>
<td>Treponema denticola</td>
<td>Forward: TAAATCGGAGTAGTGCCTAATTACAT  Reverse: TCAAGAAGCGATTCCCTTCTTCATT</td>
<td>60</td>
<td>316</td>
</tr>
</tbody>
</table>
tis, linear gingival erythema, HIV-associated periodontitis, acute ulceronecrotic gingivitis, necrotizing stomatitis, oral candidiasis, coated tongue, hairy leukoplakia and Kaposi sarcoma) was also monitored.

Statistical analysis
Data were analyzed using a statistical package (SPSS version 20.0, SPSS Inc, Chicago, IL, USA). Chi square, Student’s t-tests and Mann-Whitney U test were used to compare the prevalence of periodontal pathogenic bacteria and evaluate the differences in periodontal parameters between the study groups. A p-value smaller than 0.05 was used to assign statistical significance. The age-related occurrence of the seven considered periopathogenic bacteria was studied by establishing a six year age interval, \( a \pm 3 \), around an age value, \( a \). The age values were taken to be 26, 28, 30, 32, 53, 55, 57, 59, 61, 63, 65, and 67. The values 32 and 53 were obtained from the upper limit of the younger group, 35, and the lower limiting age of the older group, 50, as 35 –3 and 50 +3, respectively. The youngest patient was 23 years old leading to lowest considered six-year interval centered at 26=23 +3. The interval defined by the value of 67 comprised all the patients aged over 64=67 –3. The in-between age values were taken with the two-year step size. When the described twelve overlapping groups were formed from the HIV-infected patients they contained 12, 12, 10, 11, 16, 10, 10, 9, 7, 4, 3, and 4 individuals, whereas the non-infected group had 16, 19, 19, 13, 10, 6, 4, 3, 6, 9, 12, and 12 elements in the subsets that correspond to the age values 26, 28, 30, 32, 53, 55, 57, 59, 61, 63, 65, and 67, respectively.

RESULTS
Sixty HIV+ and 60 HIV- male patients were divided into two groups according to their age: 18-35 and older than 50 years. PCR analyses of 120 patient’s buccal swabs revealed bacterial DNA in 106 samples (88.3%) - 58 samples (55%) belonged to HIV+ and 48 (45%) to HIV- patients (\( p=0.203 \)). \( P. \) intermedia and \( P. \) micros were the most frequently detected bacteria in all study groups, whereas \( T. \) denticola had the lowest detection rate. The prevalence of microorganisms was significantly higher in HIV-infected patients compared to controls, except for \( T. \) denticola (Figure 1A), whereas the difference was not significant between younger and older patients (Figure 1B), except for \( P. \) gingivalis. In addition, considering oral microflora status of HIV-versus HIV+ patients, a notable shift was found; namely the distribution of bacteria in HIV- patients (decreasing prevalence) was: \( P. \) intermedia\( \rangle \)\( P. \) micros\( \rangle \)\( E. \) corrodens\( \rangle \)\( P. \) gingivalis\( \rangle \)\( A. \) actinomycetemcomitans\( \rangle \)\( T. \) forsythia whereas in HIV+ there were rearrangements in the order \( P. \) intermedia\( \rangle \)\( P. \) micros\( \rangle \)\( E. \) corrodens\( \rangle \)\( P. \) gingivalis\( \rangle \)\( A. \) actinomycetemcomitans\( \rangle \)\( T. \) forsythia. In the HIV+ group, the distribution of bacteria species was similar among younger and older patients, though there was a statistically significant difference in

![Figure 1](image1.png)

**Figure 1** - (A) Distribution of periopathogenic bacteria between HIV-infected and non-infected patients. Significant differences were found with respect to \( P. \) intermedia \( (p=0.010) \), \( P. \) micros \( (p=0.003) \), \( E. \) corrodens \( (p<0.001) \), \( P. \) gingivalis \( (p<0.001) \), \( A. \) actinomycetemcomitans \( (p=0.013) \) and \( T. \) forsythia \( (p<0.001) \). (B) Distribution of periopathogenic bacteria between patients younger than 35 years of age and older than 50 years. A significant difference was found with respect to \( P. \) gingivalis \( (p=0.006) \).

![Figure 2](image2.png)

**Figure 2** - (A) Distribution of periopathogenic bacteria between HIV-infected and non-infected patients younger than 35 years of age. Significant differences were found with respect to \( E. \) corrodens \( (p=0.006) \) and \( T. \) forsythia \( (p<0.001) \). (B) Distribution of periopathogenic bacteria between HIV-infected and non-infected patients older than 50 years of age. Significant differences were found with respect to \( P. \)intermedia \( (p=0.002) \), \( P. \) micros \( (p=0.003) \), \( E. \) corrodens \( (p<0.001) \), \( P. \) gingivalis \( (p=0.002) \) and \( T. \) forsythia \( (p<0.001) \).
the percentage of *P. intermedia* (*p*=0.010), *E. corrodens* (*p*=0.032) and *P. gingivalis* (*p*=0.008) positive patients. In non-infected subjects, the difference in bacterial distribution between younger and older participants was not significant. Most importantly, when comparing HIV+ and HIV- patients in their respective age groups, a remarkable difference in microorganisms was noted in the group over 50 years (Figure 2B). This difference was less pronounced in the younger group (Figure 2A).

The percentages of positive tests in the twelve considered six-year-wide age subsets are presented in Figure 3 separately for each of the seven bacteria types. Considerable variations in bacterial detection rate between the established age groups, in both HIV-infected and non-infected patients were registered. It is also worth noting that, when going towards older age, some bacteria showed a trend of increasing difference in distribution (*P. intermedia* and *E. corrodens*) between HIV+ and HIV- patients (Figure 3). The relationship between periodontal parameters and patient’s age is depicted in Figure 4. All measured values of standard clinical periodontal parameters (PI, PPD, CAL and BOP) with the exception of PPD, showed statistically significant differences between older HIV+ and HIV- patients. In addition, all parameters were significantly higher in older compared to younger HIV-infected patients, whereas such a difference was inexistent in the control group.

 Oral manifestations characteristic of HIV infection were noted in 17 younger patients (57%) and 27 older patients (90%). The most frequently detected oral changes were HIV-associated gingivitis, linear gingival erythema, HIV-associated periodontitis, acute ulceronecrotic periodontitis and coated tongue. Necrotizing stomatitis, oral hairy leukoplakia and Kaposi sarcoma were not present (Table 3).

As expected, the HIV+ group showed a statistically significant difference in infection and therapy duration between younger and older participants, while viral load and CD4 count were similar in the two groups, which means that the infection was maintained under control in both age groups.

**DISCUSSION**

There is growing evidence linking periodontal pathogens with serious systemic diseases and conditions such as coronary heart disease, especially atherosclerosis, diabetes, preterm delivery, infections of central nervous system, etc. (Li et al., 2000; Perunovic et al., 2015; Preshaw et al., 2012; Pucar et al., 2007). Since HAART converted HIV infection from a lethal to a chronic disease, the HIV-infected elderly population is expected to surge (Carosi and Torti, 2004). To better appreciate changes that potentially occurred in the oral cavity of HIV-infected individuals in the course of time and in comparison to non-infected persons, a cross-sectional study was designed comprising two groups of HIV positive and two groups of HIV negative patients, with a 15 year age gap between them.

We found that HIV infection considerably augments the bacterial burden in the oral cavity and alters patterns of microbiota composition in the process of aging. The recorded phenomena are most probably due to a very complex relationship between HIV infection, antiretroviral therapy and ageing. The incidence of periodontal pathogens was higher in both younger and older HIV+ patients compared to their non-infected counterparts. This difference was even more pronounced between older HIV+ and older HIV- individuals, with a trend of gap widening between the two groups. This study is the first to hypothesize that oral microflora alterations are signs of accelerated aging in HIV+ individuals.

In our study *P. intermedia* was detected as the most prevalent microorganism, a finding in agreement with other investigations on non-infected and HIV-infected subjects (Brito et al., 2008; Li et al., 2014). Besides being the prevalent microorganism, *P. intermedia* was significantly more present in the group of HIV+ compared to HIV-, and in older HIV+ compared to younger HIV+. *P. intermedia* serves as a primary colonizer of oral keratinocytes and a fundamento for secondary colonizers, such as *P. gingivalis*. *P. intermedia* is designated as a potent periodontal pathogen responsible for aggressive forms of periodontitis, acute necrotizing ulcerative gingivitis and some severe systemic infections (Dorn et al., 1998; Falkler et al., 1999). *Peptostreptococcus micros* plays an important role in the pathogenesis of periodontal disease (Haffajee and Socransky, 1994) and tends to co-aggregate with *P. gingivalis* and...
other bacteria in oral biofilm in the interest of enhanced adhesion and stability of the bacterial superstructure (Kremer and van Steenbergen, 2000). Few studies have described the presence and possible pathogenic role of *P. micros* in HIV infection (Nakou et al., 1997; Navazesh et al., 2005).

We also found a highly significant increase in bacteria of the "red complex"—*P. gingivalis* and *T. forsythia* in HIV+ compared to HIV- patients, which is basically in agreement with some previous studies (Gatto et al., 2014; John et al., 2012; Pereira et al., 2014). This finding is of great importance since it appears that *P. gingivalis*, via its metabolites, may be involved in the progression of HIV infection (Imai et al., 2012). Consequently, early treatment of periodontitis in HIV+ individuals is of utmost importance.

The main virulence factors of *Tannerella forsythia* are proteases which degrade iron-carriers and ensure amino acids and heme essential for bacterial growth (Sharma, 2010). Proteases also cleave immunoglobulins and other protective protein molecules thus affecting host immunity and providing a safe place for *T. forsythia* and other bacteria (Potempa and Pike, 2009). *A. actinomycetemcomitans* was also more frequently encountered in HIV+ than in HIV- patients, though on the incidence scale it dropped from 4th position in HIV- subjects, to 6th position in HIV+.

The prevalence of *E. corrodens* increased going towards older age, but dropped steeply in mid-sixties patients of both non-infected and HIV-infected groups. This could be due among others, to microelement disturbances accompanying both aging and HIV infection, i.e. to altered microorganisms "competition settings", especially in terms of iron and manganese availability. Higher frequencies of *T. denticola* in older HIV- compared to older HIV+ patients may be the consequence of the excessive iron intake by other bacteria, and HIV itself, leaving small amounts of iron available.

Though HIV-infected individuals have experienced a remarkable improvement in life expectancy in the HAART era, they remain at substantially higher risk of morbidity and mortality than the general population and the introduction of antiretroviral treatment has resulted in a series of disorders within the oral cavity (Greenspan et al., 2001; Nittayananta et al., 2010; Peacock et al., 2017). Thus, the higher rates of oral ulcerations, local hyperplasia of epithelium, salivary gland disease and xerostomia found in the modern HAART era could understandably interfere with microbiome status and microbial hierarchy (Navazesh et al., 2005; Soares et al., 2012). Our study showed that periodontal tissue deterioration was more frequent in older HIV+ patients compared to older controls. Periodontal status of younger HIV+ and HIV- patients appeared rather similar.

HIV-associated gingivitis and periodontitis were significantly more frequent in patients over 50 years old. Only

**Table 3 - Oral manifestations characteristic for HIV-infected patients.**

<table>
<thead>
<tr>
<th>Variable (%)</th>
<th>≤35 years n=30</th>
<th>≥50 years n=30</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-associated gingivitis</td>
<td>56.7</td>
<td>90.0</td>
<td>0.007</td>
</tr>
<tr>
<td>Linear gingival erythema</td>
<td>16.7</td>
<td>6.7</td>
<td>0.424</td>
</tr>
<tr>
<td>HIV-associated periodontitis</td>
<td>13.3</td>
<td>46.7</td>
<td>0.010</td>
</tr>
<tr>
<td>Ulceroncrotic periodontitis</td>
<td>0</td>
<td>3.3</td>
<td>1.000</td>
</tr>
<tr>
<td>Necrotizing stomatitis</td>
<td>0</td>
<td>0</td>
<td>/</td>
</tr>
<tr>
<td>Coated tongue</td>
<td>0</td>
<td>6.7</td>
<td>0.492</td>
</tr>
<tr>
<td>Hairy leukoplakia</td>
<td>0</td>
<td>0</td>
<td>/</td>
</tr>
<tr>
<td>Kaposi sarcoma</td>
<td>0</td>
<td>0</td>
<td>/</td>
</tr>
</tbody>
</table>

**Figure 4 - Distribution of periodontal parameters over age.** The following parameters using the best linear fits were observed: plaque index (PI), probing pocket depth (PPD), clinical attachment level (CAL) and bleeding on probing (BOP). Statistically significant differences in all periodontal parameters were noticed between younger (≤35 years) and older (≥50 years) HIV-infected patients, namely, PI (p<0.001), PPD (p=0.011), CAL (p<0.001) and BOP (p<0.001). The differences in periodontal parameters between younger (≤35 years) and older (≥50 years) non-infected patients were not statistically significant.
linear gingival erythema, an early stage of oral alterations, was more frequent in the younger group. The percentages of oral manifestations were in general agreement with literature data (Leao et al., 2009; Pakfetrat et al., 2015).

Poor oral hygiene is a common clinical observation in HIV-infected patients. Despite a clear necessity for oral health care, these patients are rarely provided with adequate dental treatment due to HIV-related stigma still present in many environments, which puts them automatically at higher risk for developing oral and systemic diseases (Jones et al., 2012; Patton et al., 2003). Furthermore, medical conditions accompanied by HIV infection such as lipodystrophy, nausea, vomiting, etc. often require dietary modifications (di Sibio et al., 2008; Dragović et al., 2017). Certain rearrangement of oral flora could be the response to changes in food intake and dietary habits.

In summary, deterioration of host immune system and alteration of oral tissues facilitate adhesion and invasion of pathogenic microorganisms which in turn might exacerbate the clinical course of the main disease and cause the development of other systemic diseases. Reengineering microbial ecology could potentially help to restore normal immune functioning. Further studies are needed to understand complete microbiome dynamics and to set new treatment approaches.

Conflict of interest
The authors declare there are no conflicts of interest.

Acknowledgments
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References


