

PERIODONTAL CONDITIONS AND DISTRIBUTION OF PREVOTELLA INTERMEDIA, PORPHYROMONAS GINGIVALIS AND AGGREGATIBACTER ACTYNOXYCETEMCOMITANS IN HIV-INFECTED PATIENTS UNDERGOING ANTI-RETROVIRAL THERAPY AND IN AN HIV-SERONEGATIVE GROUP OF THE VENEZUELAN POPULATION

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ABSTRACT

The aim of this study was to determine the periodontal conditions and the distribution of *Prevotella intermedia*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in a group of HIV-infected patients undergoing anti-retroviral therapy (HAART) and in an HIV-seronegative group. The study sample comprised thirty-two (32) HIV positive patients distributed in two groups (11 HIV+ without HAART and 21 HIV+ with HAART) and 16 HIV seronegative patients. Plaque index, gingival index, pocket depth, and clinical attachment level were evaluated at six sites per tooth in all teeth. Subgingival plaque samples were collected from one tooth per quadrant with pocket depth > 4 mm and attachment level > 5 mm. and then analyzed by PCR. The mean value of PI, GI, and CAL of the HIV-infected patients undergoing or not HAART- and the control group were similar; the PD

index was higher in the control group. LGE was observed only in the HIV-infected group and NUP in the HIV+ without HAART therapy. The control group and the total HIV-infected patients showed similar CPG and CPL values. *P. intermedia* was the most frequently recovered microorganism in all the groups evaluated. The second pathogen with higher prevalence was *A. actinomycetemcomitans*, *P. gingivalis* was observed only in one (5%) HIV+ patient under HAART and in three patients (19%) in the control group. The periodontal index was not related with the CD4+ count and viral load. Changes observed in the periodontal tissues of patients infected with HIV are similar to those observed in HIV negative subjects

Key words: periodontitis, HIV, HAART, periodontopathogens, CD4+.

CONDICIONES PERIODONTALES Y DISTRIBUCIÓN DE PREVOTELLA INTERMEDIA, PORPHYROMONAS GINGIVALIS Y AGGREGATIBACTER ACTYNOXYCETEMCOMITANS DE PACIENTES INFECTADOS POR VIH BAJO TERAPIA ANTIRETROVIRAL Y HIV SERONEGATIVOS EN UNA POBLACIÓN VENEZOLANA

RESUMEN

El propósito de este estudio fue determinar las condiciones periodontales y la distribución de *Prevotella intermedia*, *Porphyromonas gingivalis* y *Aggregatibacter actinomycetemcomitans* en pacientes que portan HIV bajo terapia antiretroviral de alta actividad (HAART) y un grupo HIV seronegativo.

Treinta y dos (32) pacientes VIH seropositivos distribuidos en dos grupos (11 HIV+ sin terapia HAART y 21 HIV+ con terapia) y 16 sujetos seronegativos diagnosticados con enfermedad periodontal. La evaluación periodontal se realizó registrando el índice de placa (IP), el índice gingival (IG), la profundidad de los sacos (PS) y el nivel clínico de inserción (IC). Estas medidas fueron realizadas en seis sitios por diente en todos los dientes. Muestras de placa subgingival fueron colectadas en un diente por cuadrante con bolsas > 4 mm y pérdida de inserción > 5 mm para posterior análisis por PCR.

Los valores promedio del IP, IG e IC de los pacientes VIH+ con o sin HAART y el grupo control fueron similares. El

valor promedio de la profundidad al sondaje fue mayor en el grupo control. Eritema lineal gingival (ELG) fue observado sólo en el grupo VIH+ y Periodontitis ulcerosa necrosante (PUN) en el grupo VIH+ sin terapia HAART. Los valores para periodontitis crónica generalizada (PCG) y periodontitis crónica localizada (PCL) fueron similares para los tres grupos. *P. intermedia* fue el microorganismo detectado con mayor frecuencia en todos los grupos evaluados, seguido por *A. actinomycetemcomitans* y *P. gingivalis* fue observado solo en un paciente (5%) VIH+ y tres (19%) del grupo control. Los índices periodontales en el grupo HIV+ no se relacionaron al conteo CD4+ y la carga viral. Los cambios observados en los tejidos periodontales de los pacientes que portan VIH fueron similares a los observados en el grupo seronegativo.

Palabras clave: periodontitis, HIV, HAART, periodontopatogenos, CD4+.

INTRODUCTION

Oral and periodontal lesions are common in patients infected with human immunodeficiency virus (HIV). A broad range of periodontal diseases has been reported in HIV-infected individuals including both common and less conventional forms of gingivitis and periodontitis¹. Information on the epidemiology, microbiology and natural history of periodontal conditions associated with HIV-infection is limited partly due to the absence of reliable diagnostic criteria²⁻⁶. In addition, changes in medical therapy due to the introduction of protease inhibitors and anti-viral drugs have modified the frequency and the severity of oral manifestations in HIV-infected patients. Since the application of Highly Active Anti-retroviral Therapy (HAART), clinical and epidemiological observations show a decline in the mortality and morbidity of HIV seropositive subjects, which can be attributed to a significant reduction of HIV viral load and recovery of immune function⁷.

At present, the recommended treatment for HIV infection is HAART, which consists of a combination of at least 3 antiviral drugs, 2 NRTIs with either protease inhibitors (PIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs)⁷. With the advent of the era of HAART, additional studies are being conducted to determine the epidemiology of periodontal disease in HIV infected patients.

The reported prevalence of oral lesions related to HIV is mostly from studies prior to HAART therapy⁸⁻¹⁰. Several case studies suggest improvement of existing oral lesions in HIV seropositive subjects undergoing HAART^{7,11,12}. However, there are not many studies correlating the prevalence of periodontal conditions and the significance of periodontal diseases among HIV-infected patients with or without antiretroviral therapy. Between 1980 and 1990, unusual forms of gingival erythema and necrotizing periodontal diseases were described in HIV infected individuals, which were in contrast to the conventional periodontal diseases observed in non-HIV-infected individuals. The workshop on periodontal diseases in HIV-infected individuals held at the "Fourth International Workshop on Oral Manifestations of HIV Infection", considered that the periodontal diseases commonly associated with HIV are similar or modified presentations of diseases observed in non-infected populations¹³. The data from a controlled study of relative periodontal

attachment loss in individuals with HIV infection showed no evidence of greater periodontal destruction associated with HIV infection¹⁴.

In relation to periodontal pathogens associated with periodontal disease in HIV-infected patients, studies suggest that the periodontal microflora of disease sites in the HIV infected population is similar to the periodontal microflora in non-HIV infected patients with periodontal disease¹⁵, but the combination of certain periodontal pathogens may be responsible for chronic periodontitis in HIV-infected adults¹⁶.

The aim of this study was to determine the prevalence of the periodontal conditions and the distribution of *Prevotella intermedia*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (before *Actinobacillus actinomycetemcomitans*) in a group of HIV-infected patients undergoing anti-retroviral therapy (HAART) attending the Center of Oral Infectious Diseases at the School of Dentistry, Central University of Venezuela.

MATERIAL AND METHODS

Thirty-two (32) HIV positive patients attending the Center of Oral Infectious Diseases, School of Dentistry, Central University of Venezuela, and 16 HIV seronegative patients exhibiting some form of periodontal disease according to the criteria of the 1999 World Workshop for classification of periodontal disease, attending the Periodontology Clinic, were included in this study. The HIV positive patients were stratified based on HIV therapy as HIV without HAART (11 patients) and HIV-positive with HAART therapy (21 patients), two drugs based on nucleoside reverse transcriptase inhibitors (NRTIs) and a drug based on non-nucleoside reverse transcriptase inhibitors (NNRTIs) or protease inhibitors (PIs). Patients were undergoing this therapy for a minimum of six months before they were enrolled in this study.

The immunological status and viral load of the HIV-infected group were evaluated and classified according to their CD4⁺ cells counts and plasma viral load: CD4⁺ cells > 500/mm³, 200-500 CD4⁺ cells/mm³, and CD4⁺ cells < 200/mm³. (1) Undetectable viral load < 400 copies/mm³, <10.000 viral copies and > 10.000 viral copies. Exclusion criteria included history of periodontal therapy or use of antibiotics 6 months prior to entering the study.

The study was approved by the Human Review Board of the School of Dentistry, Central University of Venezuela (UCV). All patients determined eligible signed a consent form, prior to entering the study. All patients received a periodontal examination including assessment of plaque index¹⁷, gingival index¹⁸, pocket depth, and clinical attachment level performed at six sites per tooth in all teeth. Measurements of probing pocket depth and clinical attachment level were performed by a calibrated examiner using a Williams calibrated manual periodontal probe (PQ-OW, Hu-fridey Instrumental Co) by. Subgingival plaque samples were collected from one tooth per quadrant selected as having pocket depth > 4 mm and attachment level > 5 mm. All 4 interproximal sites (mesiobuccal, distobuccal, mesiolingual and distolingual) of the test teeth were sampled (to obtain enough sample material). Supragingival plaque was removed using sterile cotton rolls and subgingival plaque samples were obtained by inserting paper points into the pocket of each site and left in place for 30 seconds, and then pooled in 250 µl of saline solution. Four paper points per tooth for a total of sixteen samples from each subject were pooled for analysis. Two hundred µl per patient were boiled during 5 min to disrupt the bacteria and then stored at -70°C for PCR analysis.

The samples were centrifuged for 5 min to eliminate cells debris; 10 ml of the supernatant were then added to a tube containing the PCR mix.

PCR amplification reactions were carried out in a reaction mixture containing 200 mM dNTPs, 2.5 U Taq polymerase, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.5 mM of each microorganism specific primers: Primer AA2 (5' CTT TGC ACA TCA GCG TCA GTA CAT CCC CAA GG 3') is specific for *A. actinomycetencomitans*, producing an amplification product of 253 pb. Primer BGING (5' TAC ATA GAA GCC CCG AAG GAA GAC G 3') is specific for *P. gingivalis*, which produces an amplified fragment of 527 bp; Primer BINT (5' TCC GCA TAC GTT GCC TGC ACT CAA G 3') is specific for *P. intermedia* with an amplification product of 163 bp, 1.5mm of the Primer E, that is common to all three bacteria (5' CGT GCC AGC AGC CGC GGT AAT ACG 3') and 10⁻⁹ mM of the positive control plasmid (pPG19) in a final volume of 50 ml.

PCR cycling was carried out in an MJ Research thermal cycler. The temperature cycling conditions

(30 in total) comprised an initial denaturation step at 94°C, 10 min, denaturing at 94°C, 1 min, annealing 70°C, 1min and extension at 72°C, 1min.

In the final step, the PCR product was fully extended for 10 min at 72°C. The PCR amplified fragments were visualized in a 3% agarose gel stained with ethidium bromide. If the clinical samples contained the microorganisms, the PCR products were 163, 253 and 527 bp length for *Pi.*, *Aa.* and *Pg.*, in addition to 1430 bp of the positive control amplification product (Pharma-gen).

This multiplex PCR assay is able to detect a minimum of 100-1000 bacteria. The specificity of the selected primers was assured by using highly stringent conditions in the PCR reaction (70°C for the annealing temperature).

Statistical analysis

Analysis of Varianza (ANOVA) was performed to determine the statistical significance potential differences between the control group and the HIV+ patients. The Kruskal-Wallis test for variables that are not normally distributed was applied. To determine the relationship between the variables the Gower index was used. To determine the differences between the percentages the test of the differences of proportions was used. The statistical analysis was done with Excel for Windows XP. The parametrical and no parametrical ANOVA were done with the statistical program Statistica V 5.5 for Windows, and the analysis of the Gower Index was done with MVSP V 3.0 for Windows.

RESULTS

Thirty-two (32) HIV positive patients 5 females and 27 males and 16 HIV seronegative patients, 11 females and 5 males, were included in this study (Table 1). The HIV positive patients were stratified according to HIV anti-retroviral therapy as HIV with HAART (21) and HIV without HAART (11). Seventy six percent (16/21) of the individuals in the HIV+ HAART group were male. All of the HIV+ patients without HAART were male (11/11). In the control group, 69% (11/16) were female and 31% (5/16) were male. The mean age of the HIV-positive group was 37.09 ± 9.10 years and the mean age of the control group was 41.62 ± 8.10 years (Table 1).

Regarding the distribution of HIV-infected patients according to the immunological conditions and peri-

Patients	N	Gender	
		F	M
Total HIV	32	5	27
HIV+HAART	21	5	16
HIV+No HAART	11	-	11
Control	16	11	5

F: Female - M: Male

odontal indexes in the present study, there were no statistically significant differences between HIV+ patients with CD4 count < 200 cells/mm³ and CD4 > 200 cells/mm³ (Kruskal-Wallis test). However, a slight difference in PD index was observed between the control group and the HIV+ group (Table 2).

The viral load average of HIV-infected patients undergoing anti-retroviral therapy was, 68.850±184.725 RNA/mm³ and 170.190±189.934 RNA/mm³ in those patients without treatment. Statistically significant differences were not observed between both groups.

The evaluation according to mean value of PI, GI, PD, and CAL of the HIV-infected patients with or without HAART and the control group, showed that the periodontal indexes (PI, GI, CAL) were similar in the total HIV-infected patients and the control group, except for the PD index which was higher in the control group (p<0.005).

The HIV-infected patients undergoing antiretroviral therapy showed slightly higher scores of GI than HIV-infected patients without therapy. Regarding to PI, PD and CAL mean values, the Kruskal-Wallis test showed and no significant differences between the HIV+ groups evaluated (Table 3).

The prevalence of periodontal lesions in HIV-infected patients undergoing HAART and with-

	PI X±S.D.	GI X±S.D.	PD X±S.D.	CAL X±S.D.
HIV+<200 11/32 34.3%	1.52±0.38	1.49±0.5	2.70±0.39	3.23±0.83
HIV+>200 21/32 65.6%	1.35±0.33	1.61±0.43	3.14±0.62	3.45±0.81
Control	1.20±0.36	1.55±0.39	3.64±0.62	3.29±0.73

PI: Plaque Index - GI: Gingival Index - PD: Pocket Depth
CAL: Clinical Attachment Loss

out HAART compared to the control group is showed in table 4. All the patients had some forms of periodontal disease, 9% of these presented Localized Chronic Periodontitis, and 64% exhibited Generalized Chronic Periodontitis. Additionally, some of these patients showed other periodontal conditions, including Lineal Gingival Erythema and Necrotizing Ulcerative Periodontitis in some localizations. LGE was observed only in the HIV-infected group, and no differences were between the groups with and without HAART. NUP was observed only in the HIV+ group without HAART.

The control group and the total HIV-infected patients showed similar CPG and CPL values. However, PIG levels were observed in a higher proportion of the control group as compared to HIV-infected patients with or without HAART therapy.

Regarding the prevalence of the three subgingival microorganisms examined in the subgingival plaque samples from the HIV-infected patients and control group. *P. intermedia* was the most frequent-

	n	PI (X±SD)	GI (X±SD)	PD (X±SD)	CAL(X±SD)
HIV+		1.4 ± 0.34	1.56 ± 0.45	3 ± 0.58	3.3 ± 0.69
HIV+ no HAART therapy	11	1.45 ± 0.3	1.41 ± 0.42	3.1 ± 0.72	3.5 ± 1.0
HIV+with HAART therapy	21	1.38 ± 0.36	1.64 ± 0.45	2.9 ± 0.51	3.3 ± 0.69
Control	16	1.22 ± 0.36	1.55 ± 0.39	3.6± 0.62*	3.8 ± 0.73

*p<0.005 compared to HIV+
PI: Plaque Index - GI: Gingival Index - PD: Pocket Depth - CAL: Clinical Attachment Loss

TABLE 4. Proportion of patients with periodontal lesions in the study groups

Periodontal lesions	HIV+ no HAART	HIV+ HAART	Control
LGE	36% (4/11)	38% (8/21)	0
PIG	9% (1/11)	19% (4/21)	44% (7/16)
LCP	9% (1/11)	48% (10/21)	44% (7/16)
GCP	64% (7/11)	52% (11/21)	56% (9/16)
NUP	27% (3/11)	0	0

LGE= Lineal Gingival Erythema
 PIG= Plaque-Induced Gingivitis
 LCP= Localized Chronic Periodontitis
 GCP= Generalized Chronic Periodontitis
 NUP= Necrotizing Ulcerative Periodontitis.

ly recovered microorganism in all the groups evaluated, 73 % in the HIV+ HAART, 76 % in the HIV+ no HAART and in all the patients of the control group ($p < 0.005$). The second pathogen with higher prevalence was *A. actinomycetemcomitans* with a frequency of detection of 50% in the control group, 33% in the HIV+ HAART and 18% in the HIV+ no HAART ($p < 0.005$) groups. *P. gingivalis* was observed only in one (5%) HIV+ patient under HAART and in three patients (19%) in the control group.

The microorganism *P. intermedia* was the only one detected in all the groups. However the highest frequency corresponded to the HIV+ non HAART group (55%) ($p < 0.005$), while *A. actinomycetemcomitans* and *P. gingivalis* were not observed alone in the groups evaluated.

The coinfection of *A. actinomycetemcomitans* and *P. gingivalis* was detected in all the groups, with higher prevalence in the control group (50%) ($p < 0.005$), while the combination of *P. gingivalis* with *P. intermedia* was observed only in 5% of the HIV-infected patients under HAART.

Additionally, the combination *A. actinomycetemcomitans* with *P. gingivalis* and *P. intermedia* was found exclusively in the control group.

DISCUSSION

HIV-associated periodontal lesions may be categorized as: unusual forms of gingivitis (termed linear gingival erythema (LGE)); necrotizing diseases (necrotizing ulcerative gingivitis (NUG), necrotizing ulcerative Periodontitis (NUP)); and chronic Periodontitis with an increased rate of attachment loss. These lesions are significant in the extent to

TABLE 5. Distribution of species detected in HIV-positive and HIV-negative patients

Bacteria	HIV+HAART	HIV+non HAART	Control
Aa	-	-	-
Pg	-	-	-
Pi	38%(8/21)	55%(6/11)	32%(5/16)
Aa + Pi	33%(7/21)	18%(2/11)	50%(8/16)
Pg + Pi	5%(1/21)	-	-
Aa + Pg + Pi	-	-	19%(3/16)

which they mark the underlying HIV disease and have service planning implications¹⁹.

These conditions appear to be same diseases as seen in HIV negative patients. However, the initiation, progression and presentation may be modified by HIV infection⁶.

The aim of this study was to determine the prevalence of the most frequent forms of periodontal disease in HIV patients and investigate the relationship between the severity of the periodontal disease and systemic markers of HIV infection progression such as viral load, CD4+ counts and HAART therapy.

Eighty four percent (84%) of the populations in the HIV+ group were male and 16% were female, in the control group 69% were female and 31% male. A high prevalence of males was observed in the HIV+ group. Similar results were reported by Myint et al (1999)²⁰, Eyeson et al (2001)²¹, Bendick et al (2002)²², while one study from Thailand (Khongkuntian et al 2001)²³ reported a higher prevalence of women.

The mean age of the patient in this study was 37.09 years in the HIV-infected patients and 41.6 years in the control group. These results are consistent with other studies^{22,24}.

In this study, no statistically significant differences were observed in clinical parameters (PI,GI,PD and CAL) and the CD4+ cells count between the HIV+ infected group and the control group. No differences were observed between the patients with anti-retroviral therapy and those without this treatment. These results suggest that the amount of periodontal tissue loss in HIV-infected patients is not related with the immunological state, in keeping with data reported by Holmstrup et al (1998)²⁵ and Alpagot et al (2004)¹.

However, in a 20-month longitudinal study published in 1992, Barr et al.²⁶, reported an increase in

attachment loss in immunodeficient individuals ($CD4^+ < 200$ cells/mm³) who were older than 35 years of age. The difference found between Barr's study and our study may be explained by the fact that protease inhibitors were not commonly used in the treatment of HIV+ subjects prior to 1992. Another study by Robinson et al (1996)²⁷, found that the decrease in CD4+ counts was correlated more with probing attachment loss but not with pocketing. However, in a later study comparing the progression of periodontal destruction in individuals infected or not with HIV, the data did not show evidence of greater periodontal destruction associated with HIV infection¹⁴. The lack of significance of the differences between the levels of CD4+ and clinical measurements could be a result of the limited sample size in these studies. The periodontal diseases frequently associated with HIV infection include linear gingival erythema, necrotizing ulcerative gingivitis and Periodontitis, and chronic Periodontitis associated with increased attachment loss²⁸. In this study 38% of the patients infected with HIV showed LGE and no differences were observed between the group under antiretroviral therapy and the group without therapy. NUP was diagnosed in three of the HIV infected patients (9%) without HAART and was not observed in the control group. These figures are higher than those reported by Kroidl et al.²⁹; who reported 9% of LGE and 3,6% NUG and NUP. These latest were found predominantly in patients with advanced immunosuppression and elevated viral load. Others reported that the prevalence of HIV-related gingival and periodontal disease varied from 0 to 47% in adults, and from 0 to 20% in children. However, not all of the studies specify the type of gingival and periodontal disease observed. When it is specified, linear gingival erythema has prevalence rates of 0-11.9% in adults with HIV infection, similarly to our results, although a prevalence of 47% has been described in India³⁰.

Linear gingival erythema has been associated with a CD4+ count < 200 cells/mm³ but not with high viral load, while NUP disease has been found to have no significant association with low CD4+ counts or high viral load³¹. These findings are similar to our results in which no relation was observed between the CD4+ counts, the viral load and NUP. Chronic periodontal disease has been described as more common and/or more aggressive in HIV-

infected patients. The occurrence of specific periodontal disease has been observed in some, but not all, groups of HIV-infected patients, suggesting perhaps that HIV alone does not predispose patients to pocketing, attachment loss or bleeding on probing^{14,32}. These observations are in accordance with our results.

Several researchers^{33,29,34} demonstrated a significant decrease in the prevalence of oral lesions in subjects on any HAART (30%) compared with the prevalence in subjects not on therapy (46%). Similar findings were documented in the cohort study carried out by Patton et al.^{35,36}; who reported that oral lesions were detected in 37.5% of the 271 HIV-infected patients, of whom 76% were undergoing HAART, compared with 47.6% in 299 patients where only 13% were receiving HAART.

A question that remains unanswered is whether changes in patients with HIV infection are specific to the infection or are a modified form of periodontal disease seen in non-infected populations¹⁶. Knowledge of the microbiology associated with these changes may help answer this question. Information on the microbiota associated with periodontitis in HIV-positive patients is controversial.

Several studies have shown that *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens* and *Campylobacter rectus* may appear in diseased sites of HIV-positive patients and HIV-seronegative subjects with classical periodontitis^{37,38}. One study reported that *P. gingivalis*, *P. intermedia*, and *Treponema denticola* appeared more frequently and in larger numbers in HIV-infected patients than in HIV-negative subjects³⁹. Some studies have shown that there is a similar flora in both groups, and no difference in the distribution of black pigmented anaerobes^{32,1}. Patel et al. (2003)¹⁶ reported a significant prevalence of *P. gingivalis* and *Treponema denticola* among HIV-negative patients compared to HIV infected patients.

In this study the non-HIV-infected patients presented higher prevalence of the microorganisms tested compared to HIV-infected subjects. The prevalence found in the groups evaluated, considering both the control and HIV-infected group were: *P. intermedia* followed by the coinfection of *P. intermedia* with *A. actinomycetemcomitans*, which was higher in a control group. Additionally *P. gingivalis* was detected only in three of the subjects that belonged to the

control group and one of the HIV infected patients, while the coinfection of these three microorganisms was only observed in the control group. Similar results were reported by Goncalves et al. (2007)⁴⁰. Regarding the effect of the antiretroviral treatment on the microorganisms evaluated, the results of this study demonstrated that the presence of these pathogens was more frequent in the patients undergoing antiretroviral treatment, suggesting than in those without any treatment that could suggest, that the HAART does not play an important role in the microorganisms population.

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