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Original Article

In situ FoxP3+ and IL-10 over-expression is associated with high grade anal lesions in HIV infected patients

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Abstract: Human papillomavirus (HPV) is the main etiologic agent of lower genital tract cancers. The natural history of HPV infection and the immune response to HIV/HPV co-infection, particularly in the anal mucosa, is poorly understood. The aim was evaluate the *in situ* immune response in anorectal biopsies from HIV-infected patients. A total of 114 biopsies were analyzed by Tissue Micro-Array: 15 were from HIV-negative individuals with normal squamous epithelium and 99 from HIV-positive individuals (21 normal squamous epithelium, 39 with anal intra-epithelial lesion grade 1 and 39 with anal intra-epithelial lesion grade 2/3). PCR and sequencing were used to identify HPV DNA. Staining for CD4, CD8, Foxp3+, T-bet and IL-10 were analyzed via immunohistochemistry. HIV-positive patients with AIN 2/3 showed a lower number of CD4+ cells (< 50 cells/mm^3) compared to HIV negative subjects (P = 0.01). HIV infected individuals showed a higher expression of FoxP3+ and IL-10 that correlated with the severity of the lesion (P = 0.002). A positive coefficient correlation was found between FoxP3+ and IL-10 (r = 0.34; P = 0.027). HPV DNA was detected in 93.4% (101/107) of the samples and the most common types were HPV 16 (26.9%), followed by HPV 6 (15.7%), HPV 59 (13%) and HPV 18 (10.2%). Our results showed a strong association between the increased T-reg cells and IL-10 expression in HIV-positive patients with AIN 2/3. HPV 16 was the most prevalent type. Our study suggests that the immune regulatory *in situ* profile may favor HPV persistence in HIV-positive individuals. Further *in situ* studies should be done in order to elucidate the development of anal cancer in HPV/HIV co-infected individuals.

Keywords: HPV/HIV-1, anal intra epithelial neoplasia, immune response, genotype

Introduction

HIV-1 infected individuals have a higher incidence, prevalence and persistence of HPV infection. This is attributed to an increased susceptibility to infection and a decreased ability to eliminate HPV via the immune response, due to a deficiency of the CD4+ T cells [1].

Regulatory T cells (T-regs) can be responsible for mediating the tolerance of the immune response by regulating the Th1 and Th2 responses. One mechanism of T-regs-mediated immune tolerance is production of IL-10 and TGF-β1. IL-10 can impair antigen presentation by dendritic cells (DC), and thus prevent T cell-mediated immune response. IL-10 inhibits the inflammatory response and contributes to the development of malignant lesions in HIV-infected individuals by down regulation of expression of Th1 cytokines and triggering a Th2 cytokine pattern [2].

Prospective studies are required to better clarify the cellular immune response in HIV-1/HPV co-infections, and other factors, that may contribute to progression to HPV-associated anal cancer. We hypothesize that HIV-induced expression of an inflammatory immune response favors HPV persistence and consequent development of anal intraepithelial lesions. The aim of the present study was to evaluate the im-

mune response "*in situ*" in anorectal biopsies from HIV-infected patients followed at the Evandro Chagas National Institute of Infectious Diseases/FIOCRUZ, RJ, Brazil.

Materials and methods

Study population, tissue samples and data collection

The study subjects comprised 52 HIV-infected women, 41 HIV infected men, and 14 men without HIV infection. The HIV infected women were drawn from a cohort established in 1996. The HIV-infected men were drawn from a cohort established in 2010. HIV-negative men were recruited from studies for testing strategies for HIV prevention. The study material consisted of anorectal biopsies obtained from the subjects at baseline, as well as follow-up biopsies from 12 subjects, obtained between 1 and 5 months after the baseline biopsy. All patients were followed up in order to monitor the development of the lesion.

Biopsies were evaluated by two expert pathologists and classified as Low-Grade anal intraepithelial neoplasia (AIN 1), High Grade anal intraepithelial neoplasia (AIN 2/3), or no evidence of dysplasia.

Socio-demographic, clinical and behavioral variables were obtained from the database of the cohort studies at INI-FIOCRUZ-RJ [3]. The following socio-demographic variables were selected: age at the time of the biopsy and sex. The Behavioral variables were: smoking, illicit drug use, history of anal sex, number of sexual partners in the last 6 months for women, and in the last 12 months for men and number of sexual male partners in the last 12 months for men. The HPV related variables were: history of anal lesions, history of cervical lesions and history of treatment for HPV-associated lesions.

The variables related to HIV were: time since HIV was diagnosed; CD4+ nadir, defined as the lowest CD4+ value; CD4+ level at the time of the biopsy (current); HIV-1 viral load (detectable at > 49 copies/IU); use of high activity antiretroviral therapy (HAART) defined as two or more analog reverse transcriptase inhibitors and one nucleoside reverse transcriptase inhibitor, a non-nucleoside analog or at least one protease inhibitor; and duration of treatment with HAART.

Written informed consent for participation in the study was obtained from all participants in strict compliance with the ethical guidelines involving human subjects in Brazil as required in the Resolution n.466/2012 of the National Health Council. The study was approved by The Institutional Review Board (IRB) of Evandro Chagas National Institute of Infectious Diseases (INI)-Oswaldo Cruz Foundation (Fiocruz) (protocol CAE 0044.0.009.000-09), Rio de Janeiro, Brazil.

HPV DNA extraction and HPV genotyping of the tissues

The biopsies were stored in liquid nitrogen and embedded in Tissue Tek®. Using a cryostat, 3 sections were of 5 μm thickness were cut for DNA testing. DNA isolations were performing using the Isolation Kit Cells & Tissue Genomic Prep Mini Spin (GE Healthcare, Buckinghamshire, United Kingdom) according to the manufacturer's protocol. PCR was performed as previously described [4]. Samples with DNA concentrations lower than 3 ng/μl were subjected to thermostable amplification of whole genetic material using Phi29 DNA polymerase with the Genomyphi V2 DNA Amplification Kit (GE Healthcare, Buckinghamshire, United Kingdom).

For detection of HPV DNA, genomic material was subjected to polymerase chain reaction (PCR) for amplification of conserved regions of the L1 gene using primer pairs PGMY09/11 [5the MY09-MY11 (MY09/11)]. Samples negative for HPV in the first reaction were subjected to nested PCR reaction using the GP5 + and GP6 + primers to amplify fragments of approximately 155 base pairs as previously reported [5the MY09-MY11 (MY09/11)]. The PCR products were purified using the Kit Nucleo Spin® Gel and PCR Clean-up (MachereyNagel, Düren, Germany). Sequencing reactions were carried out with the Big Dye Terminator v 3.1 Cycle Sequencing Kit (part number 4337455; Applied Biosystems, Foster City, CA, USA) an ABI Prism 3730 Genetic Analyzer (Applied Biosystems). The sequences obtained were further analyzed using the MEGA software 6.0 [6which currently contains facilities for building sequence alignments, inferring phylogenetic histories, and conducting molecular evolutionary analysis. In version 6.0, MEGA now enables the inference

Table 1. Clinical, behavioral and socio-demographic variables (2010-2013)

Variables	HIV Status				Total
	Diagnosis-N (%)				
	HIV negative	HIV positive			
	Normal (N = 15)	Normal (N = 21)	AIN Low Grade (N = 39)	AIN High Grade (N = 39)	
Behavioral variables					
Age (Years)					
Mean (SD)	33.6 (9.5)	38.5 (8.1)	41.5 (8.5)	44.4 (9.8)	40.9 (9.6)
Median (IQR)	33.4 (26.9-39)	37.5 (31.9-47.6)	41.6 (34.3-49)	44.6 (38.7-49.8)	40.7 (33.5-48.1)
Sex of birth-Male	15 (100.0)	6 (28.6)	19 (48.7)	20 (51.3)	60 (52.6)
Current smoking	-	8 (38.1)	10 (27.8)	11 (28.9)	29 (30.5)
Lifetime illicit drug use	3 (20.0)	2 (10.0)	7 (18.9)	8 (21.1)	20 (18.2)
No of sexual partners in the past 6 months (only for women)					
0	-	5 (35.7)	2 (10.0)	5 (27.8)	12 (23.1)
1	-	9 (64.3)	17 (85.0)	13 (72.2)	39 (75.0)
2	-	-	1 (5.0)	-	1 (1.9)
No of sexual partners in the past 12 months (only for men)					
Mean (SD)	30.3 (75.4)	9.4 (12.2)	10.6 (19.1)	6.2 (8.9)	14.1 (40.8)
Median (IQR)	8.0 (4.0-20.0)	4.0 (1.0-11.0)	4.0 (2.0-10.0)	3.0 (1.0-9.5)	4.0 (1.0-10.0)
No of sexual male partners in the past 12 months (only for men)					
Mean (SD)	30.3 (75.5)	9.0 (12.4)	9.2 (19.0)	5.9 (9.0)	13.6 (40.8)
Median (IQR)	7.0 (4.0-20.0)	4.0 (1.0-10.0)	4.0 (1.0-10.0)	2.5 (0.0-9.5)	4.0 (1.0-10.0)
Variables related to HPV					
History of anal sex	10 (76.9)	8 (40.0)	15 (40.5)	14 (35.9)	47 (43.1)
Cervical lesion history	-	10 (66.7)	13 (65.0)	12 (66.7)	35 (66.0)
Anal lesion by HPV history	2 (14.3)	3 (15.0)	11 (28.9)	9 (23.7)	25 (22.7)
Treatment for HPV history	2 (14.3)	3 (14.3)	11 (28.9)	7 (18.4)	23 (20.7)
Variables related to HIV					
Time since HIV diagnostic (months)					
Mean (SD)	-	87.3 (84.8)	104.6 (73.4)	118.7 (81.5)	106.5 (79.2)
Median (IQR)	-	60.7 (17.0-142.3)	101.0 (39.5-165.9)	127.9 (35.2-198.9)	101.0 (30.2-183.7)
Nadir CD4*					
Mean (SD)	-	205.6 (125.2)	213.3 (184.1)	122.2 (117.7)	175.8 (153.7)
Median (IQR)	-	177.0 (162.0-251.0)	174.0 (52.0-337.0)	63.0 (35.0-200.0)	156.0 (45.0-251.0)
< 50	-	2 (9.5)	9 (23.1)	17 (43.6)	28 (28.3)
50-199	-	11 (52.4)	11 (28.2)	12 (30.8)	34 (34.3)
200-349	-	6 (28.6)	11 (28.2)	8 (20.5)	25 (25.3)
≥ 350	-	2 (9.6)	8 (20.5)	-	12 (12.1)

Current CD4+*	-				
Mean (SD)	-	636.4 (377.6)	536.8 (288.9)	443.5 (277.9)	521.2 (311.0)
Median (IQR)	-	533.0 (465.0-765.0)	492.0 (354.0-707.0)	392.0 (284.0-528.0)	465.0 (334.0-707.0)
< 50	-	-	1 (2.6)	1 (2.6)	2 (2.0)
50-199	-	1 (4.8)	3 (7.7)	5 (12.8)	9 (9.1)
200-349	-	4 (19.0)	4 (10.3)	9 (23.1)	17 (17.2)
≥ 350	-	16 (76.1)	31 (79.5)	24 (61.5)	71 (72.7)
HIV RNA viral load (6 months) Undetectable	-	11 (52.4)	25 (64.1)	20 (52.6)	56 (57.1)
cART exposure (months)	-	17 (81.0)	29 (74.4)	35 (89.7)	81 (81.8)

1-Ignored information (missing): Age-0%. Sex = 0.0%. Smoking = 16.7%. Use of illicit drugs = 3.5%. Anal sex History = 4.4%. Anal Cytology = 36.0%.History of cervical lesion = 53.5%. History of HPV anal lesions = 3.5%. History of previous treatment of HPV = 2.6%. Number of sexual partners in the last 6 months (for women) = 3.7%. Number of sexual partners in the last 12 months (for men) = 5.0%. Number of male sexual partners in the last 12 months (for men) = 5.0%. CD4 nadir = 0.0 %. Current CD4 = 0.0%. HIV viral load (6 months) = 1.0%. Exposure to HAART = 0.0%. Time from diagnosis HIV = 0.0%. 2-*P*-value < 0.05 in the Fisher's exact test in CD4+ nadir CD4+ and current variables. 3-SD = Standard Deviation; IQR = 25 percent-75 percent.

Table 2. Immune markers expression between normal squamous epithelium diagnoses in subjects with HIV negative serology (2010-2013)

Marker	Normal samples	Average (±)	Median (IQR)	N	*P* value
CD4 %	HIV negative	11.3 (1.5)	10.9 (8.5-14.8)	8	0.657
	HIV positive	10.4 (1.7)	10.1 (8.6-12.4)	10	
Total				18	
CD8%	HIV negative	23.3 (3.6)	27.4 (21.6-28.5)	5	0.903
	HIV positive	25.6 (4.6)	20.9 (16.5-33.1)	10	
Total				15	
FoxP3 %	HIV negative	2.6 (0.9)	2.1 (0-5.5)	10	0.127
	HIV positive	4.6 (0.9)	4.7 (2.1-6.3)	12	
Total				22	
T-bet %	HIV negative	0.5 (0.5)	0 (0-1.4)	3	0.097
	HIV positive	2.3 (0.5)	2.1 (0.2-3.2)	14	
Total				17	
IL-10 %	HIV negative	3.3 (1.6)	3.3 (1.6-4.9)	2	0.084
	HIV positive	7.3 (0.8)	6.8 (5.3-8.7)	17	
Total				19	

of timetrees, as it implements the RelTime method for estimating divergence times for all branching points in a phylogeny. A new Timetree Wizard in MEGA6 facilitates this timetree inference by providing a graphical user interface (GUI]. The sequences were submitted to BLASTn software (available http://www.ncbi.nlm.nih.gov/blast/Blast.cgiHYPERLINK "http://www.ncbi.nlm.nih.gov/blast/Blast.cgi."). We assumed that sequences with double peaks in chromatogram are suggestive to multiple infections, once that more than one nitrogenous base was inserted in the same position. Than to investigate multiple infections we used the kit High + Low Papilloma strip (Operon-Zaragoza, Spain) according to the manufacturer's protocol, that allow identify 18 types of low risk HPV and 19 types of high risk HPV.

Immunohistochemistry

Tissue Micro Array (TMA) paraffin blocks, were prepared as previously described by Pires et al [7]. Following the construction of the TMA block, 4 μm sections were cut with a microtome and placed on silane coated slides.

Immunohistochemistry was performed as previously described [8]. Briefly, 4 μm of TMA sections were prepared from a paraffin-embedded block and dehydrated, incubated in 3% hydrogen peroxide for 10 min, and then processed for 30 min for antigen retrieval (Dakocytomation, Carpintaria-USA). Sections were blocked with 10% goat serum at room temperature for 20 min and treated with specific mouse antibody. After rinsing, sections were treated with Match4 kit-biotin-conjugated antibodies for 20 min, and streptavidin immune complexes were identified with diaminobenzidine (DAB) substrate (kit Match 4, Biocare, CA, USA) and a hematoxylin stain. Sections were mounted and dehydrated under a sealed coverslip.

Antibodies used in the study were: anti-CD4+ (R&D system, Minnesota, USA) diluted 1:100; anti-CD8+ monoclonal (Dako, Glostrup-Denmark) 1:200; anti-FoxP3 monoclonal (Abcam, Cambridge-England) diluted 1:50; anti-T-bet monoclonal (Abcam-Cambridge-England) diluted 1:150; anti-IL-10 policlonal (Abcam-Cambridge-England) diluted 1:400 dilution.

For the negative control, sections were treated identically except that the primary antibody was replaced with IgG. Quantitative analysis of the positive immune cells was calculated from the percentage of positive cells in the total field and the results were confirmed by two different viewers.

Statistical analysis

Two separate analyses were performed based on HIV status and histopathological diagnosis. One comparison was between subjects without dysplasia from HIV infected and uninfected individuals, in order to analyze differences correlated to HIV infection. The second comparison was done for HIV infected individuals comparing histopathological diagnosis (normal squamous epithelium, AIN1 and AIN2/3), to analyze differences correlated to the neoplasia grade in HIV infected subjects.

Categorical variables, including sex, smoking, illicit drug use, history anal sex and HPV variables were analyzed by Chi-square test and Fisher's exact test. Continuous variables, including age, number of partners, HIV variables, and histopathological scoring of tissue

Table 3. Immune markers expression according to the histopathological diagnosis in subjects with HIV positive serology (2010-2013)

Marker	Diagnostic	Average (SD)	Median (IQR)	N	*P* value
CD4 %	Normal	10.4 (1.7)	10.1 (8.6-12.4)	10	
	Low grade	8.0 (2.1)	6.6 (4.2-9.5)	11	
	High grade	8.5 (1.0)	7.8 (4.5-12.5)	20	0.22
CD8 %	Normal	25.6 (4.6)	20.9 (16.5-33.1)	10	
	Low grade	29.4 (3.0)	25.8 (22-36.4)	17	
	High grade	30.4 (2.6)	28.9 (21.9-38.8)	24	0.47
FoxP3 %	Normal	4.6 (0.9)	4.7 (2.1-6.3)	12	
	High grade	4.1 (1)	3.4 (0-6.1)	18	
	Low grade	11.8 (2.1)	9.6 (5-15)	23	0.002*
T-bet %	Normal	2.3 (0.5)	2.1 (0.2-3.2)	14	
	Low grade	4.4 (1.6)	2.4 (0-4.9)	16	
	High grade	3.3 (0.8)	3.3 (0-6.3)	15	0.73
Total IL-10 %	Normal	7.3 (0.8)	6.8 (5.3-8.7)	17	
	Low grade	10.1 (0.9)	9.5 (7.1-12.7)	21	
	High grade	13.3 (1.5)	10.3 (8.9-14)	21	0.003*

*P-value < 0.05.

samples were analyzed by the non-parametric Kruskal-Wallis test.

We used the Spearman correlation test to analyze possible association among immune markers expression and histopathological grading of lesions. Statistical analysis was carried with SPSS 15.0 software.

Results

Study population

Among the HIV-positive patients, there were 21 samples with normal squamous epithelium, 39 with low-grade neoplasia (AIN 1) and 39 samples with high-grade neoplasia (AIN 2/3).

Demographic, clinical, sexual behavior and immune variables according to histological results are shown in **Table 1**. The age of study participants ranged from 33.5 to 48.1 years old. The average age of the HIV-infected individuals was 42 (±0.9) years old and for the non HIV-infected individuals it was 33.6 (±2.5) years old. In the HIV-positive group, the average age of individuals with normal squamous epithelium was 38.5 (±1.8) years old; in the group with low-grade AIN it was 41.5 (±1.4) years old; and in the group with high-grade AIN it was 44.4 (±1.6) years old. Thus, in the HIV-positive group we observed an increase in age as the grade of the lesion increased, which was statistically significant (P = 0.002).

Furthermore, we observed that the peripheral levels of nadir and current CD4+ cells were decreased in individuals with AIN2/3 diagnosis and there was a statistically significant difference when compared to CD4+ T cells from subjects without dysplasia (P < 0.05).

In situ expression of CD4+, CD8+, FoxP3+ T-bet and IL-10

Immunohistochemistry was applied to the TMA, however an insufficient amount of tissue was available from some subjects to perform all the analyses. Thus, for each immune marker the total number of biopsies was: 49 for CD4+, 56 for CD8+, 63 for FoxP3+, 48 for T-bet, and 61 for IL-10.

We found no statistical difference between normal squamous epithelium samples from HIV positive and HIV negative for the immune markers analyzed (**Table 2**). **Table 3** shows the scoring obtained for each immune marker analyzed among HIV-infected subjects.

CD4+ cells: The expression of CD4+ cells was evidenced by a multifocal profile in the stroma, and some positive cells were also evident in the epithelium. There was a moderate increase of CD4+ cells in the samples with normal squamous epithelium compared to the low and high grade samples. However, despite a slight increase in the number of immune stained cells there was no statistical difference between the analyzed groups.

CD8+ cells: The CD8+ cells in the stroma showed a multifocal pattern with variable numbers of positive cells in the epithelium. There was no differential staining pattern between the analyzed groups (**Figure 1A**). Also there was no statistically significant difference in the expression levels comparing the different grades of the AIN lesions.

FoxP3+: Staining for FoxP3+ also showed a positive multifocal pattern with only a few posi-

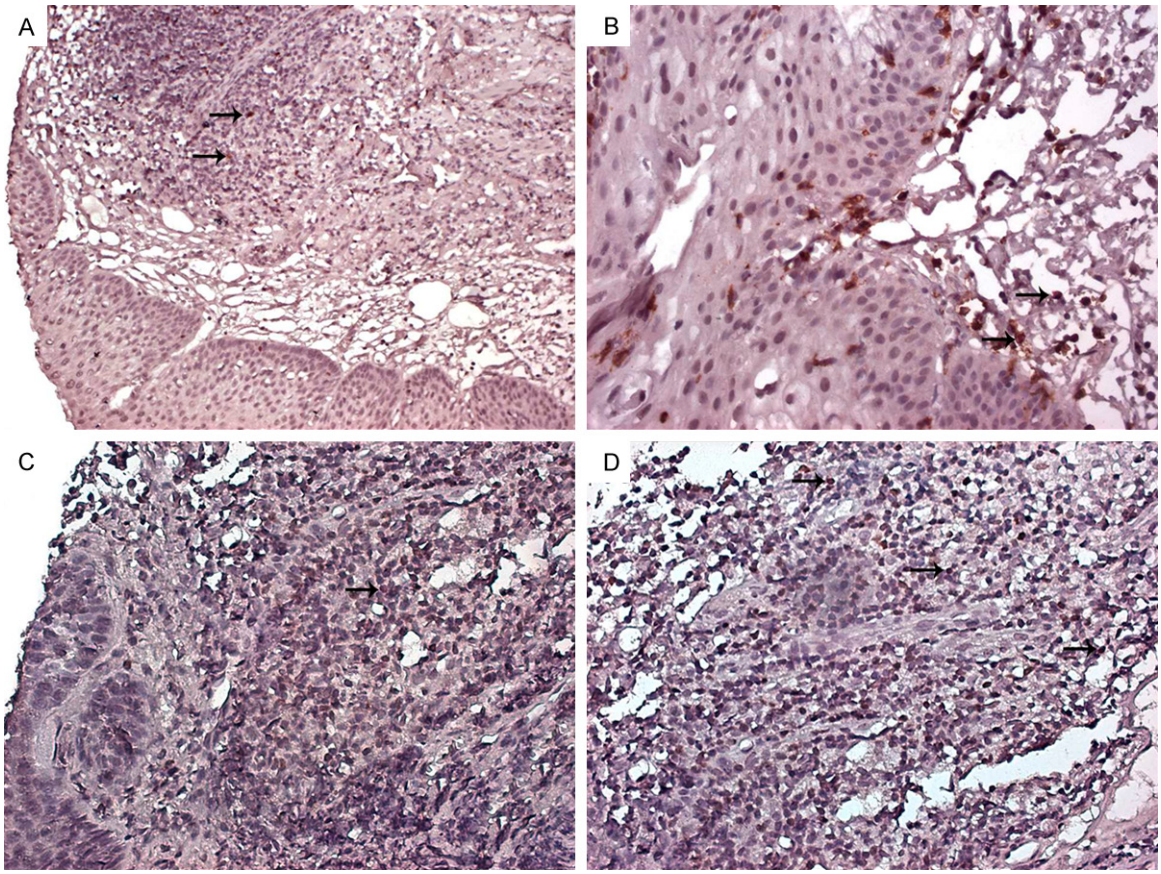

Figure 1. CD8+, FoxP3+ and T-bet immunohistochemical staining. A. Squamous anal mucosa showing moderate lymphocyte inflammatory infiltration with few lymphocytes expressing FoxP3+ in the stroma (arrow) and in the epithelium (20×). B. Squamous anal mucosa exihibiting a slight lymphocyte inflammatory infiltration with CD8+ stained cells in the stroma and epithelium (40×). C. Squamous anal mucosa showing moderate lymphocyte inflammatory infiltration with rare T-bet stained cells (AIN III) (arrow). (40×). D. Fragment and stromal anal biopsy exibithing moderate lymphocyte inflammatory infiltration with few lymphocytes expressing T-bet (arrow) (40×).

tive labeled cells in the epithelium. There was an increased number of positive cells in samples with high grade AIN compared to samples with normal squamous epithelium and low grade AIN (**Figure 1B**). Samples with normal squamous epithelium, with AIN low-grade and AIN high-grade had a mean expression of 4.6%, 4.1% and 11.8% respectively of FoxP3+ cells. This increase was statistically significant (*p*-value = 0.002).

T-bet: A focal stained profile of T-bet expression was only observed in the stroma and there was a slight increase in the number of positive cells in the group with normal squamous epithelium compared to the subjects with dysplastic lesions (**Figure 1C**, **1D**). However, we did not observe a statistically significant difference among AIN subjects and those with normal squamous epithelium (**Table 3**).

IL-10: The IL-10 expression showed a multifocal profile, predominantly in the stroma, with only a few positive cells in the epithelium (**Figure 2**). Interestingly the anal samples from HIV infected subjects had a higher IL-10 expression in high-grade AIN lesions (mean 13.3%) than in the normal squamous epithelium and low grade AIN (7.3% and 10.1%) respectively (*p*-value = 0.003).

Next we analyzed the correlation between the immune markers and CD4+ T cell counts in peripheral blood. There was a moderate positive correlation (r = 0.34; *p*-value = 0.029) between the level of expression of CD4 *in situ* and peripheral CD4+ T cell count. There was a strong negative correlation (r = -0.51; *p*-value = 0.004) between CD4 expression *in situ* and IL-10 (**Figure 3A-C**).

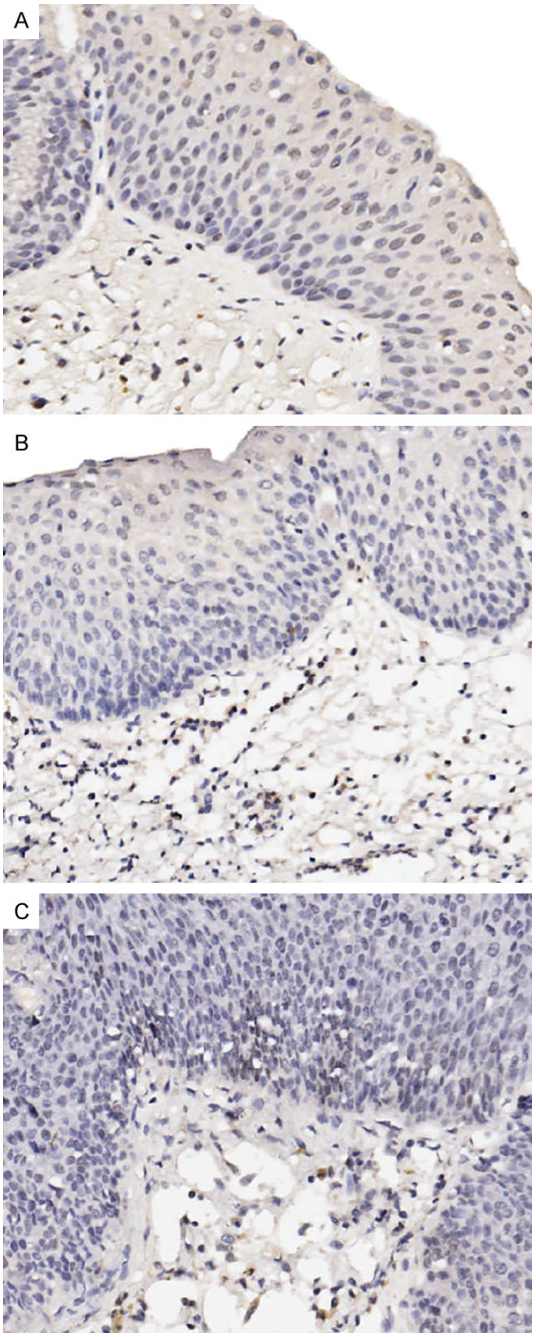

Figure 2. IL-10 immunohistochemical staining. Squamous anal mucosa from HIV infected individuals showing a multifocal profile, predominantly in the stroma, with few positive cells in the epithelium. Panel (A) shows anal tissue with normal squamous epithelium (20×) with few IL-10 stained cells (brown color) in the stroma (20×). Panel (B) Shows AIN 1 (low grade) and (C) AIN 2/3 (high-grade) exhibiting higher IL-10 expression in the stroma (20×).

There was a moderate positive correlation between FoxP3+ and IL-10 (0.34; *p*-value = 0.027) (**Figure 3D**). No statistically significant differences were found when comparing other pairs of variables. Moreover we observed a weak positive correlation between T-bet and Foxp3+ expression, with a borderline significance (r = 0:34; *p*-value = 0.055) (data not shown).

HPV typing in the biopsies

From the 114 biopsies, 7 were excluded from the analysis due to the negative result of human β-globin, leaving 107 samples for analysis. PCR showed that 93.4% (101/107) of the samples were positive for HPV DNA. The HPV negative samples were confirmed with nested PCR with the nested primer pair GP5 +/6+ and showed positive results for β-globin.

By sequencing we found eight low risk HPV types (6, 11, 40, 42, 43, 54, 61, 89); 11 high-risk oncogenic types (16, 18, 31, 33, 35, 39, 51, 52, 56, 58, 59) and 4 classified as other probable high risk types (53, 66, 67 and 69). The most common type in anal biopsies was HPV 16 with 26.9% of the samples positive for this genotype (29/107); followed by HPV 6 with 15.7% (17/107); HPV 59 with 13% (14/107); and HPV 18 with 10.2% (11/107) (**Table 4**).

A statistical difference was found (*P* = 0.02) in the frequency of HPV 6 in among AIN low grade when compared with normal squamous epithelium and AIN high grade groups. We found that 81.1% (30/37) of high grade AIN specimens were positive for high-risk HPV types compared to 57.1% (12/21) of samples with normal squamous epithelium (*p*-value = 0.03).

In addition, we found that 9.72% (9/107) of the specimens were positive for multiple HPV infections, all in HIV infected individuals. Among these specimens, one was AIN high grade, two normal squamous epithelium and six AIN low grade. Six samples were positive for HPV high and low-risk oncogenic genotypes, and three samples were positive for multiple infections with only high-risk HPV type.

Moreover, we observed that 59.8% (64/107) (data not shown) of the specimen were infected at least with one of the four HPV types covered by the HPV vaccine quadrivalente (6, 11, 16 and 18).

Discussion

HIV/HPV co-infection leads to important biological disorders, which may trigger the devel-

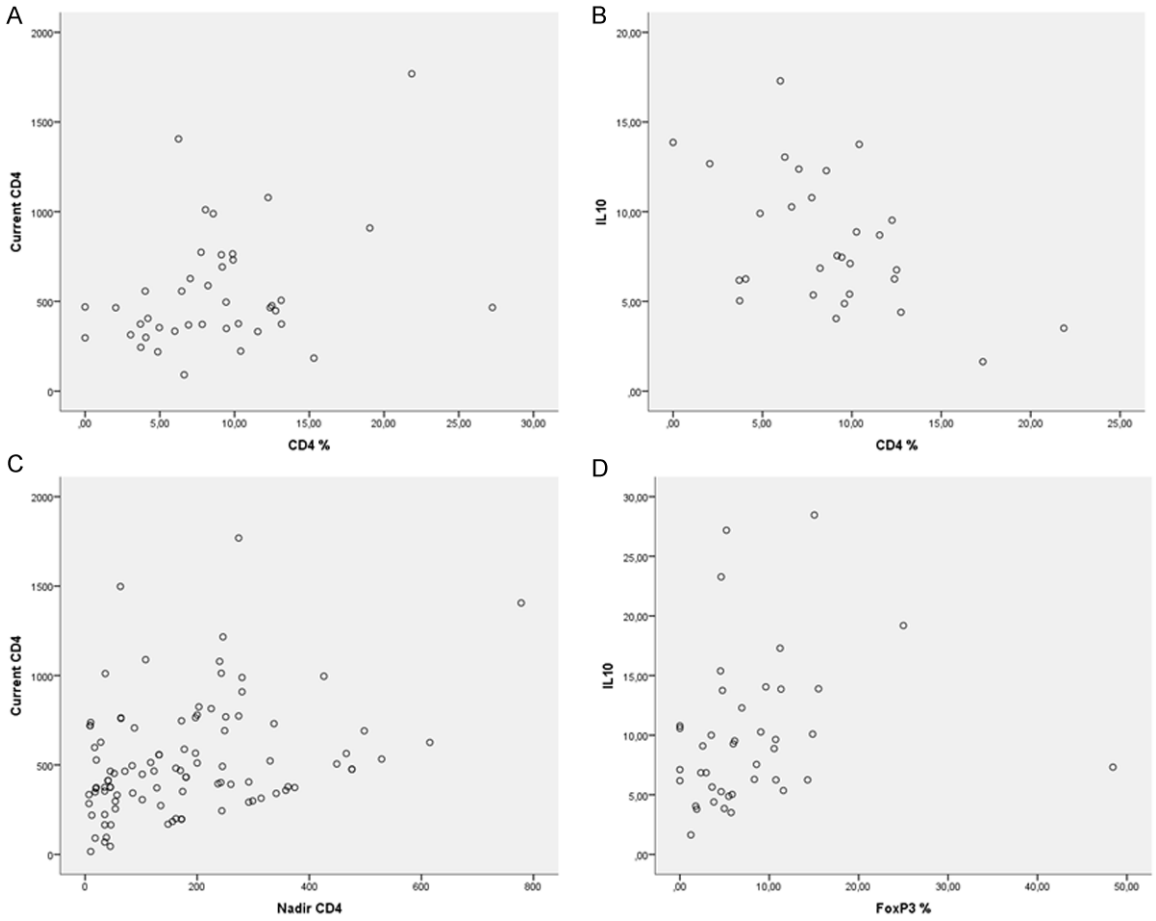

Figure 3. Correlations found. (A-C) Shows the Pearson's coefficient of correlation; (A and B) exhibit a positive correlation; and (C) negative correlation. (D) Shows the Spearman's positive coefficient correlation (2010-2013).

opment and progression of AIN to cancer. Indeed, the development of an HPV positive cancer has been considered an AIDS-defining diagnosis [9]. Most studies of the immune response to HPV infections in HIV infected patients have been done in cases of cervical cancer. Previous studies of the cervix have shown significant differences between the number of immune competent cells and the degree of malignancy, indicating that the immune response in the stroma is critical for lesion regression or progression to cervical cancer. Additionally, co-infection may lead to a shift profile from Th1 to Th2 [10].

In the present study no statistically significant difference was found in the frequency of CD4+ and CD8+ cells in AIN lesions compared to specimens with normal squamous epithelium from HIV-infected individuals. Our finding contradicts a previous study, which found a reduction of CD4+ cell numbers in the stroma of cervical intraepithelial neoplasia (CIN) lesions in HIV positive women [11].

Regarding T-bet expression, a transcription factor associated with Th1 cells differentiation, we observed weak immunohistochemical staining but no statistically significant differences were found among the different histopathological groups. However, Qinghua et al 2012 observed a higher T-bet expression in cervical cancer lesions compared to normal cervical samples [10]. In the present study we did not analyze cancer specimens.

We analyzed FoxP3+ to address the role of regulatory T cells in the anal biopsies. We observed a significant increase in Foxp3+ positive cells in the stroma samples with AIN 2/3 compared to samples without dysplasia from HIV-infected patients. Our findings are in agreement with

Table 4. HPV type detected on the anal biopsies (2010-2013)

HPV type	HIV negative		HIV positive		Total
	Normal (N = 15)	Normal (N = 21)	AIN Low Grade (N = 39)	AIN High Grade (N = 39)	
High risk					
HPV 16	3 (27.3)	3 (14.3)	10 (26.3)	13 (35.1)	29 (27.1)
HPV 18	-	4 (19.0)	4 (10.5)	2 (5.4)	10 (9.3)
HPV 31	-	-	3 (7.9)	-	3 (2.8)
HPV 33	-	-	1 (2.6)	2 (5.4)	3 (2.8)
HPV 35	-	-	2 (5.3)	3 (8.1)	5 (4.7)
HPV 39	-	-	2 (5.3)	1 (2.7)	3 (2.8)
HPV 51	-	-	1 (2.6)	-	1 (0.9)
HPV 52	-	2 (9.5)	2 (5.3)	-	4 (3.7)
HPV 56	-	1 (4.8)	-	-	1 (0.9)
HPV 58	-	1 (4.8)	1 (2.6)	4 (10.8)	6 (5.6)
HPV 59	2 (18.2)	2 (9.5)	6 (15.8)	4 (10.8)	14 (13.1)
Probable high risk					
HPV 53	-	-	1 (2.6)	2 (5.4)	3 (2.8)
HPV 66	-	1 (4.8)	1 (2.6)	1 (2.7)	3 (2.8)
HPV 67	-	-	1 (2.6)	-	1 (0.9)
HPV 69	1 (9.1)	1 (4.8)	1 (2.6)	-	3 (2.8)
Low risk					
HPV 6*	1 (9.1)	5 (23.8)	7 (18.4)	4 (10.8)	17 (15.9)
HPV 11	1 (9.1)	1 (4.8)	6 (15.8)	-	8 (7.5)
HPV 40	-	-	1 (2.6)	-	1 (0.9)
HPV 42	-	-	2 (5.3)	-	2 (1.9)
HPV 43	-	-	1 (2.6)	-	1 (0.9)
HPV 54	-	-	1 (2.6)	-	1 (0.9)
HPV 61	-	1 (4.8)	2 (5.3)	1 (2.7)	4 (3.7)
HPV 89	-	-	1 (2.6)	-	1 (0.9)
Multiple infection					
HPV 6, 16, 52	-	1 (4.8)	-	-	1 (0.9)
HPV 18, 52	-	1 (4.8)	-	-	1 (0.9)
HPV 11, 16, 18, 31, 35, 39, 52, 6, 43, 61	-	-	1 (2.6)	-	1 (0.9)
HPV 11, 16	-	-	1 (2.6)	-	1 (0.9)
HPV 6, 11, 40, 16	-	-	1 (2.6)	-	1 (0.9)
HPV 18, 33, 39	-	-	-	1 (2.7)	1 (0.9)
HPV 11, 54, 31, 59	-	-	1 (2.6)	-	1 (0.9)
HPV 11, 61, 59	-	-	1 (2.6)	-	1 (0.9)
HPV 69, 16	-	-	1 (2.6)	-	1 (0.9)
Negative HPV	3 (27.3)	2 (9.5)	-	2 (5.4)	7 (6.5)
HPV oncogenicity					
Low risk	2 (18.2)	6 (28.6)	10 (26.3)	5 (13.5)	23 (21.5)
High risk*	6 (54.5)	12 (57.1)	23 (60.5)	30 (81.1)	71 (66.4)
High/Low risk	-	1 (4.8)	5 (13.2)	-	6 (5.6)

*P-value < 0.05. Missing: HPV genotypes = 6.1%, HPV oncogenicity = 6.1%.

previous studies which saw an overexpression of FoxP3 in cervical cancer samples, AIN and anal cancer. These findings suggest that the high number of FoxP3+ positive cells in the anal

mucosa can inhibit anti-tumor immune response, probably via a TGF-dependent pathway [12]. Furthermore, high levels of T-regs in the anal tissue cells of patients with chronic HPV infection can delay the elimination of the virus by the immune system [13]. Another study showed an imbalance of local immunity due to local depletion of immune cells and an overexpression of Foxp3+ [14]. Several studies suggest that an increase of T-regs in mucosa is a characteristic of HIV infection, especially in untreated individuals indicating that these cells have an important role in the pathogenesis of the disease [14].

Th2 immune response was evaluated by IL-10 detection. We observed statistically significant differences (p-value = 0.003) when comparing the three histopathological groups among HIV-positive samples with elevated IL-10 in the AIN2-3 lesions. IL-10 is a cytokine that may inhibit the inflammatory response of the host in order to promote the development of tumor cells. Therefore, an increased secretion of anti-inflammatory cytokines such as IL-10 decreases an antitumor response [15]. This activity confirms the data found in our previous study where there was an increase of this cytokine in cervical cancer samples and CIN in HIV-infected patients [16]. In addition, an increase of IL-10 is also related to HPV's ability to escape the immune surveillance system, since this cytokine impairs antigen-specific T cell responses. This is in accordance with studies that indicate that the prevalence of Th2 cytokines in lesions caused by HPV is associated with the development of neoplastic lesions [17]. We also observed a positive correlation between IL-10 and Foxp3+ expression in HIV infected samples, suggesting that the high grade lesions (AIN 2/3) of these patients have an immunosuppressed microenvironment. In agreement with our data, another study found an increased expression of these two proteins in neoplastic lesions associated with HPV.

HPV DNA was detected in 93.51% of the total anal biopsies analyzed. Our data was in agreement with a recent review article that found a prevalence of 92.6% of anal HPV DNA in women and 80-90% in men [18].

Several studies of AIN samples with HIV-infected individuals found HPV 16 as the most common type which is similar to what we observed in this study [19]. The present study found HPV 6 was the second most prevalent HPV type and HPV 18 the fourth most prevalent type. Our results corroborate a previous study that found HPV 6 widely detected in low-grade anal lesions.

Several studies indicate that anal warts are usually considered benign and are not associated with high-risk HPV [20]. Furthermore, we found that 9.72% of the samples in the HIV-infected individuals had multiple infections. Another study also showed a high prevalence of multiple HPV infection in the anal canal of MSM, who are a risk group for the development of anal cancer [21].

Most patients who were diagnosed with low and high grade AIN reported history of receptive anal sex and high number of recent sexual partners. This feature is consistent with other AIN studies, where receptive anal sex history, number of sexual partners and smoking are considered risk factors for both men and women for the development of AIN and anal cancer, as well as cervical cancer for women [22, 23]. We found a statistically significant difference (p-value = 0.002) in age of patients with high grade AIN (44.4 years) compared to those without dysplasia (38.5 years) in the HIV infected group. Our data are in agreement with a recent study which found a mean age of 42 years in women with AIN associated with HIV/HPV [23]. Our data are in agreement with a recent study which found a mean age of 42 years in women with AIN associated with HIV/HPV [23].

The present study had some limitations, such as the relatively small number of specimens among the different histopathological groups and the HIV infected and uninfected individuals, limiting subgroup analyses. However, our study highlighted important and new data regarding the inflammatory immune response due to the HIV/HPV infection in the anal dysplasia, since the literature is still limited in this area.

Additionally we observed that more than 59% of the HIV-infected individuals have already been exposed to the 4 HPV types covered by the current quadrivalent HPV vaccine, suggesting that the HPV vaccination should be considered as a prophylactic approach to reduce the risk of anal intraepithelial lesions development in young HIV-infected individuals, especially MSM.

In conclusion, the present study suggests that the AIN2/3 (high grade lesion) of HIV-infected subjects may be related to the high prevalence of high-risk oncogenic HPV type and the high expression of immune regulatory IL-10 cytokine (Th2). This promotes an unfavorable microenvironment for viral elimination especially with the increased expression of Foxp3 (regulatory cells), which promotes persistent infection.

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Disclosure of conflict of interest

None.

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