Original Article

Coinfection of Epstein-Barr virus, cytomegalovirus, herpes simplex virus, human papillomavirus and anal intraepithelial neoplasia in HIV patients in Amazon, Brazil

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ABSTRACT: **Objective**: The prevention of anal cancer is a goal of worldwide Aids support centers. Despite the efforts that have been made and progress in the antiretroviral therapy, effective disease control remains elusive. Difficulty in preventing anal cancer may result from the ineffectiveness of highly active antiretroviral therapy on the human papillomavirus (HPV) since the coinfection with HIV and HPV appears to increase the risk of HPV-infected cells, becoming cancerous. **Methods**: We evaluated 69 HIV-positive and 30 HIV-negative male patients who underwent cytological evaluation by RT-PCR for the presence of HPV, Epstein-Barr virus, cytomegalovirus and herpes virus types (HSV) 1 and 2, and histopathology analysis of the anal canal. **Results**: The prevalence of anal intraepithelial neoplasia was 35% and it was restricted to HIV-positive patients. Patients infected with high-risk HPV and with fewer than 50 TCD4 cells/μL showed an anal intraepithelial neoplasia rate of 85.7% compared to those with TCD4 cells >200 cells/μL ($p<0.01$). The rate of viral coinfection was 16.9% of the sexual transmitted diseases cases and it was correlated with HIV-1 viral load of more than 10.001 copies/mL ($p=0.017$). The rate of AIN in coinfected patients was 36.4% ($p=0.047$). **Conclusions**: In this study, at the main institution for the treatment of HIV/AIDS in the Amazon region of Brazil, anal coinfection with HPV, cytomegalovirus, HSV-1, HSV-2 and Epstein-Barr virus occurred only in HIV-positive patients and it was directly influenced by the viral load of HIV-1. In this study, anal viral coinfection showed no additional risk for the development of anal intraepithelial neoplasia.

Keywords: sexually transmitted disease; anal coinfection; human papillomavirus; Epstein-Barr virus; cytomegalovirus; herpes simplex virus; anal intraepithelial neoplasia; anal cancer.

RESUMO: **Objetivo**: A prevenção do câncer anal tem sido aplicada pelos centros de apoio a pacientes com Aids em todo o mundo. Apesar dos esforços empregados, o eficaz controle da doença permanece distante. A dificuldade na prevenção do câncer anal pode resultar, em parte, da ineficácia da ação da terapia antirretroviral sobre o papilomavírus humano (HPV), pois a coinfecção com HIV e HPV parece aumentar o risco das células infectadas pelo HPV em tornarem-se cancerosas. **Métodos**: Foram avaliados 69 HIV-positivos e 30 pacientes HIV-negativos do sexo masculino, que foram submetidos à avaliação citológica anal por *real time*-PCR para a presença de HPV, vírus Epstein-Barr, citomegalovírus e herpes vírus tipos (HSV) 1 e 2 além da análise histopatológica de fragmento de mucosa do canal anal. **Resultados**: A prevalência de neoplasia intraepitelial anal foi de 35% e foi restrita a pacientes HIV-positivos. Os pacientes infectados com

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o HPV de alto risco e com contagem inferior a 50 células TCD4/μL mostraram taxa de neoplasia intraepitelial anal de 85,7%. A diferença foi significativa quando comparado a pacientes com células TCD4 >200 células/μL (p<0,01). A taxa de coinfecção viral foi de 16,9% dos casos de doenças sexualmente transmissíveis e diretamente correlacionada à carga viral HIV-1 superior a 10,001 cópias/mL (p=0,017). A taxa de neoplasia intraepitelial anal em pacientes coinfectados foi de 36,4% (p=0,047). **Conclusões**: Neste estudo, realizado na principal instituição para o tratamento de HIV/Aids na região amazônica do Brasil, a coinfecção anal com HPV, citomegalovírus, HSV-1, HSV-2 e vírus Epstein-Barr ocorreu somente em pacientes HIV-positivos e foi influenciada pela carga viral do HIV-1. Neste estudo, a coinfecção viral anal não representou risco adicional ao desenvolvimento da neoplasia intraepitelial anal.

Palavras-chave: doenças sexualmente transmissíveis; coinfecção anal; papilomavírus humano; Epstein-Barr vírus; citomegalovírus; herpes simples vírus; neoplasia intraepitelial anal; câncer anal.

INTRODUCTION

The human papillomavirus (HPV) is the most prevalent sexually transmitted disease (STD) of the perianal region[1,2], and it is associated with a broad spectrum of benign and malignant lesions[3]. In HIV-positive men, who have sexual relations with men, there is frequent association between multiple HPV types[3], increasing the risk of anal intraepithelial neoplasia (AIN) and anal cancer by at least 60.1 times[4].

Among immunosuppressed patients, cytomegalovirus (CMV) has been established as a cause of anorectal disorders[5], ulcers, vesicles and perianal warts, which are often associated with herpes simplex virus[6]. Coinfection by multiple pathogens is not uncommon among immunocompromised patients. Davis and Goldstone[7] described 86% of STD in patients with proctitis, and three cases of multiple infections. The association between viral and bacterial coinfections has also been described[8].

The presence of coinfections appears to increase the risk of HPV-infected cells progressing to malignancy. These pathogens act synergistically to induce anogenital epithelial lesions, particularly in HIV-positive patients[9]. The Epstein-Barr virus (EBV) infection has also been studied in genital warts and perianal intraepithelial lesions for which the role of synergism in the etiology of cancer has also suggested[10,11].

Developing a more detailed understanding of the relationship between viral coinfections and the development of anal cancer, particularly in HIV-positive patients, is of critical importance. This study aims to assess the prevalence of viral coinfection with AIN in the main institution for the treatment of HIV/AIDS in the Amazon region of Brazil.

METHODS

Ninety-nine male patients were evaluated from July, 2010 to June, 2011. Sixty-nine patients were HIV-positive and 30 were HIV-negative, who had no known risk factors for anal cancer. This study received authorization from the Research Ethics Committee. Volunteer patients, who met the eligibility criteria, were submitted to the collection of cytological material for the detection of EBV, HPV, CMV, HSV-1 and HSV-2 as well as anal inspection, digital rectal examination, anoscopy magnification imaging, and biopsies of the suspected areas. If there were no injuries or clinical suspicion, a biopsy was performed at the three o'clock position along the dentate line.

Hybrid capture was used to detect the main types of HPV with either low or high oncogenic potential[12]. The samples were processed according to the manufacturer's recommended protocols. Levels of HPV 16 ≥1 pg/mL relative lights unit (RLU) were designated as positive.

Molecular diagnoses of HSV-1, HSV-2, CMV and EBV were performed by real-time PCR (RT-PCR) using the 7500 Fast Real-Time PCR System, manufactured by Applied Biosystems. The samples were extracted with a QIAamp DNA Mini Kit according to the manufacturer's instructions. H1P32/H1M32, H2M40/H2P4, HSV-GF/CMV-R and HSV-GF/EBV-R primers were purchased from Invitrogen Life Technologies with Maxima® SYBR Green/ROX qPCR Master Mix (2X). The cycling conditions were 50°C for two minutes, 95°C for ten minutes, 40 cycles at 95°C for 30 seconds and 60°C for one minute[13].

In this study, to calculate rates of coinfection anorectal, isolated HPV-HIV infection was not considered.

RESULTS

Overview

In this study, the mean age of HIV-positive patients was 34.3 years-old; the mean age in the Control Group (HIV-negative) was 47.4 years-old (p<0.001). The average duration of HIV infection was 44 months, and the average under treatment with highly active antiretroviral therapy (HAART) was 34 months. The prevalence of AIN in HIV-positive patients was 35%, and AIN was absent in the Control Group (p<0.001) as determined by histopathological analysis. 45.8% (n=11/24) of patients presented with low-grade AIN (AIN I), and 54.2% of the patients presented with high-grade AIN (AIN II/III), with p<0.001, (data are not shown).

The impact of STDs

Patients were evaluated for the presence of a STD, either alone (n=65) or in the form of coinfection (n=11). Twenty-three patients, all HIV-negative, did not show any STD. The rates of STD infections were as follows: low-risk HPV, 73.8% (n=48); high-risk HPV, 90.8% (n=59); HSV-1, 7.7% (n=5); HSV-2, 16.9% (n=11); EBV, 7.7% (n=5); CMV, 1.5% (n=1) –data not shown. Of the patients with at least one type of STD, 57 out of 65 were HIV-positive (p<0.01). The term 'HPV total' was defined as an infection with either high or low oncogenic potential HPV, which occurred in 60.6% (n=60) considering all patients and in 81.2% (n=56) of HIV-positive patients (RR=26.6; p<0.001). Similar results were found in the analysis of HPV oncogenic risk (OR=29.5 and 26.2 for the occurrence of HPV infection, high and low risk in HIV-positive patients, respectively, n=97; p<0.001, Fisher's exact test) – data not shown.

The prevalence of AIN in HPV infected patients

Patients were evaluated to assess the influence of HPV infection on the occurrence of AIN. It was found that 86.4% (19/22) of patients diagnosed with AIN were infected with low-risk HPV (OR=9.81; p<0.001), and 95.8% were infected with high-risk HPV (OR=23.7; p<0.001), as in Table 1. Patients without HPV infection were not present with AIN (n=39), as seen in Table 2; all patients with severe AIN

Table 1. *The association of AIN with oncogenic HPV.*

HPV Types	No AIN		AIN present		Total		
	n	%	n	%		RR	p-value
Low HPV							
No	46	61.3	3	13.6	49		
Yes	29	38.7	19	86.4	48	9.81	<0.001
Total	75	100	22	100	97		
High HPV							
No	38	51.4	1	4.2	39		
Yes	36	48.6	23	95.8	59	23.7	<0.001
Total	74	100	24	100	98		
Total HPV							
No	39	52.0	0	0.0	39		
Yes	36	48.0	24	100.0	60	-	<0.001
Total	75	100	24	100	99		
HPV association							
No	46	61.3	6	25.0	52		
Yes	29	38.7	18	75.0	47	4.68	0.001
Total	75	100.0	24	100.0	99		

HPV: human papillomavirus; Total HPV: low-risk HPV or high-risk HPV infection; HPV association: infection by both low- and high-risk HPVs; data were analyzed using Fisher's exact test.

(n=13) were infected with high-risk HPV, and the rate of association between high- and low-risk HPV was 83.3% (p<0.001 for all analyses in Table 1). To assess the potential for a single infection with low-risk HPV to cause AIN, 39 patients with AIN, who were found to be negative for high-risk HPV, were analyzed; and only one of these patients was positive for low-risk HPV and was infected with low-grade squamous intraepithelial lesion – LSIL (data not shown).

Patients with severe AIN (n=13) began the study presenting a median duration of HIV-1 infection of 54 months (with a range of 3 to 144 months); 76.9% of the patients (n=10) were using HAART and showed a significant increase in the average duration of HAART use compared to patients with normal histology or inflammatory or mild AIN (p=0.041), as seen in Table 2.

The influence of HIV-1 viral load and TCD4 cell concentration

Patients infected with HPV had a significantly higher viral load of HIV-1 than those not infected (41,540 and 17,693 copies/μL, respectively; p=0.03) (data not shown). We also evaluated the potential of CD4 + T cells of causing AIN in patients infected with HPV-H. In this analysis, patients infected with high-risk HPV with TCD4 cell concentrations of <50 cells/μL presented AIN at a rate of 85.7% (n=6), as follows: four cases of AIN III and two of AIN I. Of the patients with high-risk HPV infection and levels of TCD4 cells >250 cells/μL, 70.3% did not present AIN (n=22; p<0.01), as seen in Table 3.

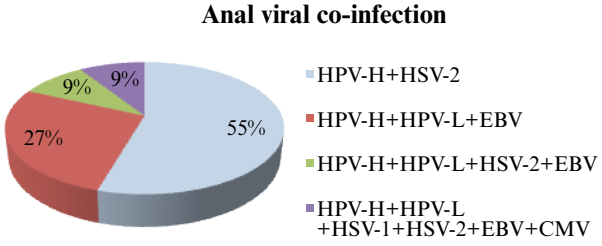
Viral coinfection

We evaluated the occurrence of anal viral coinfection, which was detected in 11 of 65 patients (16.9%) with a STD. Of the 11 coinfected patients, 6 (55%) presented a coinfection of high-risk HPV with HSV-2; 3 (27%) were coinfected with both low- and high-risk HPV and EBV; 1 (9%) patient presented with a triple coinfection of low- and high-risk HPV, HSV-2 and EBV; and the last case was coinfected with all pathogens tested (Figure 4). 100% (n=11) of anal coinfections occurred in HIV-positive patients (p=0.01) and 60% in man who has sex with man (MSM).

Table 2. *The relationship between AIN severity and the duration of HAART use.*

Duration of HAART use	Histopathology		p-value
	NEG AIN (+) AIN I	AIN III	
Average	2.23	5.34	0.041
Standard deviation	2.59	6.3	
Median	0.82	2.84	
n	27	10	

AIN: anal intraepithelial neoplasia; HAART: highly active antiretroviral therapy; data were analyzed with Mann-Whitney test.



HPV: human papillomavirus; HSV: herpes simplex virus; EBV: Epstein-Barr virus; CMV: cytomegalovirus.
Figure 4. *Rates of anal viral coinfection.*

Table 3. *The occurrence of AIN in relation to the peripheral levels of TCD4 cells in HIV-positive individuals infected with high-risk HPV.*

TCD4 (cells/μL)	AIN				p-value
	No AIN	AIN I	AIN III	Total	
<50	1	2	4	7	<0.01
>250	22	5	4	31	
Total	23	7	8	38	

AIN: anal intraepithelial neoplasm; HPV: human papillomavirus; data were analyzed using Fisher's exact test.

Table 5. *Histopathological studies of coinfected patients.*

Histopathology	Coinfection								
	0 coinfect		1 coinfect		2 coinfect		4 coinfect		Total
Histo	n	%	n	%	n	%	n	%	
Normal	20	23.8	0	0.0	0	0.0	0	0.0	20
Proctitis	34	40.5	4	44.4	0	0.0	0	0.0	38
AIN I	7	8.3	2	22.2	1	100.0	0	0.0	10
AIN III	12	14.3	0	0.0	0	0.0	1	100.0	13
No neo.	9	10.7	3	33.3	0	0.0	0	0.0	12
Metaplasia	2	2.4	0	0.0	0	0.0	0	0.0	2
Total	84	100	9	100	1	100	1	100	95

AIN I: low-grade anal intraepithelial neoplasia; AIN III: anal intraepithelial neoplasia of moderate or high degree; no neo: other non-neoplastic-specific processes such as condyloma, herpes or tuberculosis; coinfect: coinfection; 0 coinfection: no coinfection; 1 coinfection: one coinfection occurred; 2 coinfection: two coinfections occurred; 4 coinfection: four coinfections occurred.

The average TCD4 cell concentration in coinfected patients was 141 cells/μL and 35,500 copies/μL of HIV-1 viral load. There were significant differences found when comparing patients to a viral load of either less than or greater than 10,000 copies/μL (p=0.01) (data no shown). In patients with viral load lower than 10,000 copies/μL, there were only simple coinfections; in the group with higher viral loads, there were eight patients with coinfections: six are cases of simple coinfection, one case of triple coinfection, and another patient infected with all four types of investigated STDs. There was no difference in the duration of HAART use in HIV coinfected patients (p=1.0 and p=0.81, respectively) (data not shown).

AIN and coinfection

Among the 11 coinfected patients, there were four cases of AIN (36.4%), one of AIN III and three of AIN I. Therefore, no correlation was found between the incidence of AIN and viral anal coinfection (Table 5). Among coinfected patients, there were also three condyloma cases and four nonspecific inflammatory changes in these patients (Table 5).

DISCUSSION

In this study, we addressed viral coinfection in HIV-positive patients and its relationship with AIN and HAART patient and immune status. We began the analysis with an evaluation of the influence of HIV on HPV infection rates. A 26.6-fold greater risk of infection by high and/or low oncogenic HPV types in HIV-positive patients was observed. These findings corroborate those of previous studies[14-16]. Sobhani et al.[17] and Kreuter et al.[18] highlighted the greater multiplicity of high- and low-risk HPV in anal margin carcinomas compared to carcinomas of the anal canal, which are mostly associated with HPV 16.

An increased risk of severe high-grade squamous intraepithelial (HSIL) has been described in patients infected with oncogenic HPV, particularly HPVs 16 and 18, when associated with low TCD4 cell concentrations before HAART initiation (OR=14.18)[19]. These data are similar to those found in the present study in patients infected with high-risk HPV and a current TCD4 cell count of <50 cells/μL. The risk of AIN in patients with low TCD4 cell counts has also been previously reported[20,21].

A recent study described the possible protective effect of HAART on the development of severe AIN when HAART was administered for more than four years, and the analysis was adjusted for TCD4 nadir cells[19]. According to Crum-Cianflone[22], the beneficial effect of HAART may be less pronounced for individuals with moderate or severe AIN as a result of the already severe degree of HIV-related immune depletion. In those cases, either the immune restoration promoted by HAART was insufficient to restore

specific immunity to HPV-infected cells or, in cases with a more severe degree of AIN, patients may have accumulated sufficient genetic alterations to lead the failure of HAART[22]. Other authors argue that the duration of HIV infection is of primary importance, since patients infected with more than 15 years had 12 times greater chance of developing anal cancer ($p<0.001$)[23]. The average time of HIV infection in this study was much higher than that of our patients with HSIL.

Regarding the anal coinfection, we did not detect coinfection in HIV-negative subjects, probably as a result of the strict selection criteria employed for the Control Group that did not involve MSM. Anal coinfection occurred in 40% of patients who had not declared themselves adherents of receptive anal sex, highlighting the potential for the acquisition of STDs regardless of the sexual practice, as previously noted[24,25], and emphasizing the importance of prevention programs that encompass heterosexual patients.

Our rate of anal STDs are similar to the findings of Sobhani et al.[26], who described an 8% rate of EBV coinfection, a 12% rate of CMV anal infection and a 24% rate of HSV anal infection. However, this study found lower prevalence of EBV (7.7%) and CMV (1.5%) infection compared to the findings of Löwhagen et al.[27] and Rodrigues et al.[9], who reported a prevalence of HSV and EBV of over 30%. Because we used RT-PCR as a means of diagnosis, and the method is known to be higher than conventional PCR and the culturing of samples[28]; therefore, we do not consider our results to be contradictory with previous findings, but as features of our study population.

The presence of HSV-EBV coinfection as a risk factor for the development of anal cancer among patients of both sexes coinfected with HPV-HIV has already been described[9-11]. In the present study, we report five cases of coinfection with EBV. Three cases had severe AIN, and one patient was coinfected with all the viruses studied and presented very low levels of peripheral TCD4 cells. In a study conducted in Rio de Janeiro, Brazil[9], HPV-EBV coinfection occurred in 21% of HIV-positive patients and in 7% of HIV-negative patients ($p=0.017$). These data are similar to those from a study conducted in Skovde, Sweden[27], which found HSV rates similar to ours but with significantly higher percentages of EBV.

Regardless of geographical area, a considerably high rate of anal coinfection in immunocompromised patients has been found, highlighting the importance of STD surveillance and the monitoring of clinical progression in terms of AIN occurrence and anal cancer. In Botswana, Africa, an increase has been seen in ocular surface squamous neoplasia pathology with significant rates of coinfection with HPV, HSV, CMV and EBV, particularly in HIV-positive patients, but in percentages much higher than those reported here[29].

Despite the limited number of coinfected evaluated individuals and the limitations inherent in cross-sectional studies, these results show that patients with anal viral coinfection by HPV, HSV-1, HSV-2, EBV or CMV showed no additional risk for the development of AIN and that coinfection seems to be directly related to high HIV-1 viral load. New follow-up studies are needed to confirm the findings revealed in this study regarding the incidence of AIN and anal cancer in coinfected individuals.

CONCLUSIONS

This study was conducted in the main institution for the treatment of HIV/AIDS in the Amazon region of Brazil. The rate of AIN was associated with severe infection with high and low risk HPV, low levels of TCD4 cells, and a high HIV-1 viral load. Anal coinfection by HPV, CMV, HSV and EBV occurred only in HIV-positive patients, it was directly influenced by the HIV-1 viral load. In this study, viral anal coinfection showed no additional risk for the development of AIN.

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