Assessment of risk factors and human papillomavirus (HPV) related pathogenetic mechanisms of CIN in HIV-positive and HIV-negative women.

Study Design and Baseline Data of the HPV-PathogenISS Study

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Summary

Objectives: In women with HIV-associated immunosuppression, HPV infections have an increased risk of progression to high-grade cervical intraepithelial neoplasia (CIN). With the HAART-induced prolonged survival and more protracted clinical course of AIDS, progression of CIN to cervical cancer (CC) has become a clinically relevant issue, and the mechanisms responsible for HIV-HPV interactions need further elucidation. The study design and analysis of the baseline data of our new project are presented.

Material and Methods: This project is a combination of a prospective cohort study of HIV- and HIV+ women, and a retrospective analysis of CIN lesions and cervical cancer. Up to the present, 244 women have been enrolled (17 HIV+) and subjected to epidemiological interview, colposcopic examination, sampling for HPV testing and typing (PCR, InnoLiPA), and HPV serology. The retrospective series of biopsies were analysed for 13 biomarkers (monitoring key molecular events) using immunohistochemistry and tested for HPV by PCR and TaqMan.

Results: HIV- and HIV+ women differ in their exposure status to many of the key epidemiological risk factors of cervical cancer, the most significant ones being number of sexual partners ($p = 0.0001$), age at onset of sexual activity ($p = 0.002$), and contraception (yes-no) ($p = 0.009$). The differences in the baseline clinical observations are less dramatic; HIV-positive women had more frequent HSIL PAP tests ($p = 0.040$), CIN2 or higher in cervical biopsy ($p = 0.049$), and external genital warts ($p = 0.019$). The factors predicting intermediate endpoint markers of cervical cancer, i.e., HSIL PAP smear, ATZ2 in colposcopy, and high-grade CIN in biopsy were analysed in univariate and multivariate regression models. All factors significant in univariate analysis were entered in the multivariate model; HIV-status and Pap smear history maintained their independent predictive power of the HSIL Pap test. The most powerful predictor of ATZ2 colposcopy was HSIL in Pap test. Only the HSIL Pap test and ATZ2 colposcopy remained significant independent predictors of high-grade CIN ($p = 0.0001$ and $p = 0.008$, respectively) in the multivariate model.

Conclusions: The three intermediate endpoint markers are closely interrelated, but predicted in part by different covariantes in the causal pathway to cervical cancer. To elucidate whether the increased risk of HIV-positive women to high-grade CIN is due a) to their different exposure status to the risk factors, b) to the direct effects of HIV, or c) to molecular interactions between HIV and HPV, we need to complete these analyses separately in HIV+ and HIV- women.

Key words: HPV; HIV; Interaction; Risk factors; Pathogenesis; CIN; Colposcopy; Pap smear; Intermediate endpoint markers.

Introduction

Even before the link between human papillomavirus (HPV) and cervical cancer was disclosed, compelling epidemiological evidence suggested that cervical cancer bears close similarities with a sexually transmitted disease (STD). Several characteristics of sexual behaviour have repeatedly been associated with an increased risk for cervical cancer, including early onset of sexual activity, multiple sexual partners, promiscuous sexual partners, use of contraceptives, and poor sexual hygiene [1, 2]. In addition to these STD-related factors, a variety of other factors have been shown to increase the risk for cervical cancer, such as low socioeconomic status, reproductive history, smoking habits, dietary factors, history of Pap smear, and immunosuppression [1-6]. Not unexpectedly, these same factors have been convincingly documented as the most significant risk factors of HPV infections as well [1, 2].

Among the immunocompromised subjects, women with HIV infections and AIDS comprise a special group, known to be at increased risk for HPV infections and cervical intraepithelial neoplasia (CIN) [7-11]. HPV infections and CIN in these women run a fulminant clinical course, with rapid progression to high-grade CIN and cancer [12-18]. During the 1980's and early 1990's, these

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women frequently succumbed to HIV/AIDS prior to the development of an invasive cervical cancer [1-3, 19, 20]. Following the development of the highly effective antiretroviral therapy (HAART), however, the clinical course of HIV has been substantially protracted, and these women currently comprise a clinically significant group of patients with an increased risk to develop CIN and cervical cancer [7-10, 19-21].

Accordingly, HIV-infected women require special clinical surveillance of their lower genital tract to enable early detection of HPV infections and CIN [22-24]. In this surveillance, Pap smear cytology has been proven as a sensitive diagnostic tool as compared e.g., to the performance of colposcopy [7-11, 25, 26]. On the other hand, the value of HPV typing in this surveillance is under debate, and additional data are needed to establish the role of HPV testing as a screening tool in monitoring of HIV-infected women [27-33]. During the past several years, a multi-center study coordinated by the Italian National Institute of Health (ISS) (the DIANAIDS project), has explored these different diagnostic tools in the management and monitoring of HIV-infected women in Italy [8-11, 13, 25, 26].

Apart from the management of these women, the molecular interactions of the two viruses (HIV-HPV) remain largely uncovered [1-3]. Although some important in vitro data have emerged recently [34-38], it is of particular importance to assess the impact of these mechanisms on the risk and clinical behaviour of CIN in these women. A new study design (called HPV- PathogenISS project) was recently formulated to include a multi-centre prospective study and a retrospective series of CIN and CC to compare the pathogenetic mechanisms and prognostic factors of CIN lesions in HIV-positive and HIV-negative women [39]. The present report describes the study design and the analysis of the epidemiological and baseline clinical data of the prospective cohort of this new project.

Material and Methods

Patients

The present series comprises a total of 244 women at the moment, examined and enrolled by gynaecologic outpatient departments at five hospitals (S. Orsola Malpighi, Bologna; Istituto Regina Elena, Rome; Università di Tor Vergata, Rome; Ospedale Luigi Sacco, Milano; Istituto San Gallicano, Unità Operativa MST/HIV, Rome). Women enrolled in the study are among the consecutive patients referred to these second level reference centers for further examinations and treatment due to abnormalities (ASC-US, LSIL or HSIL) in a recent Pap test. The mean age of these women is 36.5 years (range: 19-82), and all have given their informed consent to participate in the study.

Methods

The outline of the prospective study is shown by the patient flowchart (Figure 1). All women are subjected to colposcopy, Pap test (if not taken within the past 3 months), and sampling for HPV testing. The further allocation of the patient into a follow-up or treatment group depends on the severity of the cervical abnormality in the punch biopsy. This study protocol was separately approved by the institutional Ethical Committees of all five hospitals.

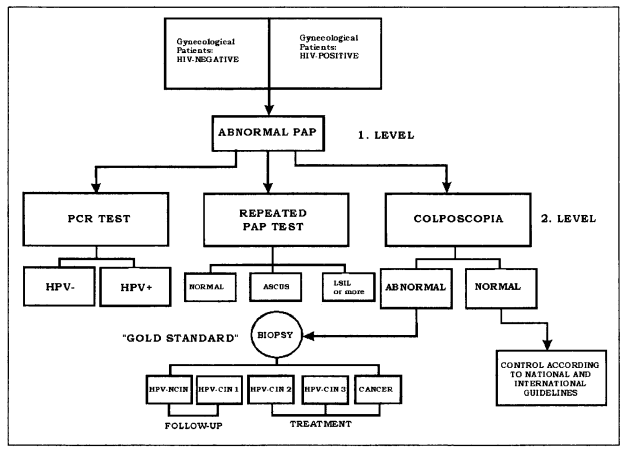

Figure 1. — Study design and flow chart of the patients.

Epidemiological interview

At the first clinical visit, every woman was interviewed for gynaecologic and obstetric history as well as for all implicated epidemiologal risk factors of CIN and cervical cancer using a structured questionnaire.

Pap test

If not taken less than three months before, the women were subjected to a confirmatory Pap smear, taken according to routine procedures, processed according to a conventional technique, and screened and interpreted using the Bethesda 2001 system (TBS 2001) [40].

Colposcopy

Colposcopic examination in these second level reference centers was performed for all women using instruments of the latest design and state-of-art procedures. All examinations were done using both acetic acid and iodine application, and the terminology used is the Italian equivalent to the 1990 Colposcopy Nomenclature of the IFCPC (41). The low-grade abnormalities are called ATZ1 (atypical transformation zone grade 1) and high-grade abnormalities ATZ2 in the Italian modification of this widely accepted nomenclature.

Biopsy

Directed punch biopsies were taken from all colposcopic abnormalities according to routine procedures. The biopsies were fixed in 10% neutral formalin and processed into HE-sections for light microscopy. On histological examination the lesions were graded using the CIN nomenclature and categorized as CIN [1-3]. The morphological evidence for HPV infection (koilocytosis, dyskeratosis, multi-nucleation, etc.) was also separately recorded in the biopsies [2]. Flat HPV lesions without evidence of CIN were called HPV-NCIN, following the previous practice [2].

Treatment by LLETZ

Following the histological confirmation of CIN2 or higher, women were treated with large loop electrosurgical excision of the transformation zone (LLETZ) [42], or any of the optional treatment modalities [2, 3].

Sampling for HPV testing

A careful sampling protocol for HPV testing was followed in all clinics. Both exo- and endocervical specimens are collected and stored at –70°C until transported (on dry ice) to the two virology laboratories (Tor Vergata and ISS) for analysis. In the laboratory the samples were transferred to an Eppendorf tube and centrifuged at 8,000 rpm for 1 min. The supernatant was removed and the pellet stored frozen at –80°C before performing the HPV test.

Polymerase chain reaction (PCR)

The pellet was resuspended in 200 µl of sterile PBS and the DNA extracted according to the manufacturer's instructions (QIAamp DNA Mini kit, Qiagen, Germany). An aliquot of 5 µl of DNA was first amplified with β-actin primers (sense: 5'-GGCGGCACCACCATGTACCCT-3', anti-sense: 5'-AGGGGCCGGACTCGTCATACT-3'). The PCR mix contained 200 µM each dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, 40 pmol sense and anti-sense primer, 1.25 U AmpliTaq Gold (Applied BioSystem, The Netherlands). The PCR conditions were: 94°C, 10 min for one cycle; 94°C, 30 sec, 60°C, 30 sec, 72°C, 30 sec for 25 cycles and finally, 72°C for 7 min. The amplicons were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualised under UV light. All samples gave the expected fragment size of 202 bp.

The samples were then screened for the presence of HPV with one round PCR using the degenerated primers MY09/MY11. The PCR conditions for the MY09/MY11 primers were: 94°C for 10 min, one cycle; 94°C, 30 sec, 55°C, 45 sec, 72°C, 30 sec for 40 cycles, followed by an extension step at 72°C for 7 min. The PCR mix contained 40 pmol each primer, 2 mM MgCl2, 1.25 U AmpliTaqGold. The amplified product was run on a 1% agarose gel, stained with ethidium bromide and visualised under UV light. This primer pair amplify a 450 bp fragment of the L1 gene [43].

HPV genotyping

Positive samples were subjected to HPV typing using primer sets specific for the E6/E7 region (Amplimedical, Torino, Italy) of the low-risk (L-R) (HPV 6/11) and high-risk (H-R) (HPV 16, 18, 31, 33, 35, 45, 52, 53, 58, 66) HPV types, respectively. The PCR conditions were the following: 94°C for 5 min, 72°C for 10 min (1 cycle), then 40 cycles at 94°C for 1 min, 55° for 1 min, 72°C 1.5 min and finally, 72°C for 5 min, 80°C for 10 min (1 cycle). The PCR sensitivity was 50 HPV genomes/reaction. The primer sets specific for L-R types amplify a 230 bp fragment, while those for the H-R types amplify a 240 bp (HPV 16, 31, 33, 35, 52, 58) and 270 bp (HPV18/45), respectively [44]. The PCR products were run on a 3% agarose gel, stained with ethidium bromide, and visualised under UV light.

HPV serology

Whole blood (venous route) was collected following strictly aseptic conditions using Vacutainer® tubes with integrated serum separator; serum was aseptically transferred to the test tubes. All sera were stored and transported at –20°C to the virology laboratory (ISS) for analysis of HPV antibodies using ELISA and VLPs (to be reported later).

HIV testing

The HIV-sero status of the patients was determined with ELISA test (Elisa - Dupont; Geneting System, Seattle, WA) and confirmed by Western blot (Bio-Rad, Hercules, CA), performed at entry (or done before) with the informed consent of the patients [8-10]. The state of HIV disease in sero-positive patients was assessed by counting the CD4+ and CD8+ lymphocytes and recording the opportunistic infections or neoplasia. All HIV-positive patients are being treated with highly active anti-retroviral treatment (HAART), using INRT-, INNRT- and PI-based regimens and any of their combinations [11].

Statistical analyses

Statistical analyses were performed using the SPSS® computer program package (SPSS for Windows, version 11.5). Frequency tables were analysed using the Chi-square test, with likelihood ratio (LR) being used to assess the significance of the correlation between the categorical variables. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated using the exact method. Differences in the means of continuous variables between the patient categories were analysed using non-parametric tests (Mann-Whitney) or the ANOVA (analysis of variance) test after careful control of the normal distribution (Kolmogorov-Smirnov test with Lilliefors correction). Logistic regression models were used to analyse the power of different covariantes as predictors of the outcome variables (HSIL in Pap, ATZ2 colposcopy, high-grade CIN) both in univariate- (crude ORs) and multivariate (adjusted ORs) analysis, using the stepwise backward approach and LR (likelihood ratio) statistic for removal testing ($p = 0.10$ for stepwise removal, and $p = 0.05$ for stepwise entry). In all tests values of $p < 0.05$ were regarded as statistically significant.

Results

Of the 244 women enrolled in the prospective cohort, 17 were HIV-positive, one was a transplant recipient, and one was uncertain (both the latter were excluded from the analyses). The key epidemiological factors recorded by questionnaires are summarized in Table 1. All 242 patients were Caucasian in origin. HIV-positive and HIV-negative women differ from each other in many of the key epidemiological risk factors of cervical cancer. The most significant discriminating factors include: number of sexual partners ($p = 0.0001$), age at onset of sexual activity ($p = 0.002$), and contraception (dichotomous variable yes-no) ($p = 0.009$). Other statistically significant ($p < 0.05$) distinguishing variables are: HIV+ women are older (limit at 35 years), more frequently current smokers, smoke more cigarettes per day, have more pregnancies (but equal number of deliveries), and show a different profile of previous genital infections, i.e., frequently other than Candida.

The baseline clinical observations of the women are summarised in Table 2. The differences between the two groups are less dramatic than those shown by the epidemiological data in Table 1, and none of these differences reaches the significance level of 1% ($p = 0.01$). Accordingly, HIV-positive women are shown to have more frequently than HIV-negative women, HSIL Pap tests ($p = 0.040$), CIN2 or higher in cervical biopsy ($p = 0.049$), and external genital warts ($p = 0.019$). Due to the limited number of HIV+ women, the power of the study to disclose true differences is not yet particularly high, however.

The factors predicting the intermediate endpoint markers of cervical cancer (i.e., high-grade lesions in Pap

Table 1. — *Epidemiological data recorded by the questionnaire.*

Characteristic	HIV-Positive (n = 17)		HIV-Negative (n = 225)		OR (95% CI)	Significance
Ethnic origin	Caucasian		Caucasian			
Age (yrs)	38.5 (± 5.9)		36.4 (± 11.4)			
Age below or above 35						p = 0.446
< 35 yrs					Reference	
> 35yrs					3.41 (1.06-10.91)	
Smoking status:						p = 0.066
Current smoker	11	73.3%	95	44.0%		
Ex-smoker	1	6.7%	15	6.9%		
Non-smoker	3	20.0%	106	49.1%		
Non- Smoker	4		121		Reference	p = 0.037*
Smoker	11		95		3.50 (1.08-11.34)	
No. of cigarettes per day	19.7 (± 11.8)		13.4 (± 7.8)			p = 0.013*
Age at start smoking	17.1 (± 2.6)		17.8 (± 4.4)			p = 0.643
Age at stop smoking	30.0		29.9 (± 7.8)			p = 0.993
Age at 1st intercourse	16.1 (± 2.1)		18.4 (± 2.5)			p = 0.002***
No. sexual partners	8.8 (± 13.5)		3.4 (± 2.2)			p = 0.0001***
No. of sexual partners						p = 0.176
0-2	2		69		Reference	
3-5	6		74		2.89 (0.54-14.32)	
> 6	4		26		5.30 (0.91-30.72)	
No. of pregnancies	1.8 (± 1.4)		0.99 (± 1.2)			p = 0.019*
No. of deliveries	0.80 (± 0.6)		0.52 (± 1.1)			p = 0.947
Age at first delivery	22.5 (± 4.2)		24.8 (± 6.0)			p = 0.346
Contraception:						p = 0.134
None	8	66.7%	59	28.6%		
Condom	0		15	7.3%		
Oral	3	25.0%	105	51.0%		
IUD	1	8.3%	10	4.9%		
Natural method	0		17			
Contraception (Y/N):						p = 0.009**
Yes	4		147		Reference	
No	8		59		4.98 (1.44-17.17)	
Genital Infections:						p = 0.047*
No	8		99			
HSV	2		4			
Syphilis	1		0			
Candida	4		102			
Chlamydia	0		2			
Genital infection (Y/N):						p = 0.442
No	8		99		Reference	
Yes	7		108		1.18 (0.64-1.95)	
Previous genital HPV						p = 0.199
No	8		141		Reference	
Yes	7		68		1.26 (0.78-2.05)	
Pap smear history:						p = 0.359
Regular	6	46.2%	125	59.0%	Reference	
Irregular	5	38.5%	75	35.4%	1.38 (0.41-4.70)	
Never	2	15.4%	12	5.7%	3.47 (0.63-19.12)	

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

smear, colposcopy and biopsy), used as outcome variables in univariate analysis are shown in Table 3. Of the covariates in the potential causal pathway, i.e., exposure preceding the outcome, the HSIL Pap test is predicted by HIV status, young age at first sexual intercourse, and Pap smear history (particularly never having taken a Pap test).

Similarly, high-grade abnormality in colposcopy (ATZ2) is predicted significantly by the HSIL in Pap test ($p = 0.0001$), Pap smear history ($p = 0.003$), and negatively by previous genital infections (mostly Candida) ($p = 0.026$). Interestingly, HIV status is not a significant predictor of ATZ2 colposcopy.

Finally, the detection of high-grade CIN (CIN2 or higher) in cervical biopsy has several significant predictors; HSIL in the Pap test ($p = 0.0001$), ATZ2 colposcopy ($p = 0.001$), HIV status and age at first sexual intercourse. Interestingly, high-grade CIN is negatively predicted by the detection of external genital warts. HPV detection data were not included in any of these analyses because they are still largely premature. Similarly, age (as a con-

Table 2. — *Baseline clinical data in the two patient categoris.*

Characteristic	HIV-Positive (n = 17)		HIV-Negative (n = 225)		OR (95% CI)	Significance
Pap Smear:						p = 0.311
N-SIL	0		0			
ASC-US	1		52			
LSIL	3		110		NC	
HSIL	4		33			
Suggesting Cancer	0		3			
High-grade Pap:						p = 0.040*
No	4		162		Reference	
Yes	4		36		4.50 (1.08-18.85)	
Histology: (n = 171)						p = 0.073
Normal	0		28	17.2%		
HPV-NCIN	1	12.5%	31	19.0%		
CIN1	1	12.5%	43	26.4%		
CIN2	3	37.5%	13	8.0%		
CIN3	2	25.0%	43	26.4%		
Microinvasive	0		2	1.2%		
SCC	0		2	1.2%		
AIS	1	12.5%	0			
Other	0		1	0.6%		
CIN2 or higher:						p = 0.049*
No	2	25.0%	104	63.0%	Reference	
Yes	6	75.0%	61	37.0%	5.11 (1.01-26.32)	
CIN3 or higher:						p = 0.429
No	5	62.5%	116	70.3%	Reference	
Yes	3	37.5%	47	29.7%	1.48 (0.34-6.44)	
Signs of HPV in biopsy:						p = 0.135
No	6	75.0%	80	48.5%	Reference	
Yes	2	25.0%	85	51.5%	0.31 (0.06-1.60)	
Abnormal colposcopy:						p = 0.785
No	4	30.8%	45	27.3%	Reference	
Yes	9	69.2%	120	72.7%	0.84 (0.25-2.87)	
HR (ATZ2) colposcopy:						p = 0.092
No	7	53.8%	125	75.8%	Reference	
Yes	6	46.2%	40	24.2%	2.67 (0.85-8.43)	
Genital condylomas:						p = 0.019*
No	6	42.9%	143	73.7%	Reference	
Yes	8	57.1%	51	26.3%	3.79 (1.30-3.62)	
HPV#:						
PCR-GP	5	35.7%	19	29.2%		p = 0.427
PCR-SPF	5	71.4%	17	34.7%		p = 0.171

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; NC = not computable; GP = general primers; SPF, SPF primers; # data preliminary.

tinuous variable) was not a significant predictor of any of these outcome variables.

To disclose the significant independent predictors of these intermediate endpoint markers, all factors that were significant in univariate analysis were entered in multivariate logistic regression models. The results are shown in Table 4, where both the crude- and adjusted ORs are included, to better evaluate the potential confounding factors. The confounding effect of age was excluded by separate testing in each model.

HIV-status and Pap smear history maintained their independent predictive power of the HSIL Pap test also in the multivariate model. Never having a Pap test was associated with HSIL with adjusted OR 5.79 (1.28-26.13), which is highly significant (p = 0.004). The most powerful predictor of ATZ2 colposcopy was the HSIL Pap test with no confounding in the multivariate model (similar crude and adjusted ORs). The other predictors remained significant, but with much less statistical power. Particularly, the Pap smear history was markedly confounded by the other factors in multivariate analysis. Despite meticulous multivariate modelling (forward and backward LR and conditional methods), only HSIL Pap test and ATZ2 colposcopy remained highly significant predictors of high-grade CIN (p = 0.0001 and p = 0.008, respectively), while all other variables were removed from the model. The adjusted ORs are even higher than the crude ORs, suggesting no confounding factors in the model. In addition, there was no interaction between these two, because the interaction term (HSIL & ATZ2) did not prove to be an independent predictor, and the ORs of the two predictors remained unchanged.

Table 3. — *Significant predictors of the intermediate endpoint markers in univariate analysis.*

Dependent variable	Predictor	Statistics*	
		OR (95% CI)	Significance
HSIL in the Pap test:	HIV status	4.50 (1.08-18.85)	p = 0.040
	Age at 1st intercourse	1.21 (1.01-1.44)	p = 0.034
	Pap history:		p = 0.086
	Regular	Reference	
	Irregular	3.58 (1.01-12.78)	p = 0.049
	Never	4.02 (1.05-15.37)	p = 0.042
	CIN2 or above		p = 0.0001
	No	Reference	
	Yes	18.18 (6.48-51.01)	
	CIN3 or above		p = 0.0001
	No	Reference	
	Yes	12.01 (5.03-28.62)	
	Colposcopy ATZ2		p = 0.0001
	No	Reference	
	Yes	5.87 (2.47-13.93)	
Significant colposcopy (ATZ2):	HSIL in Pap		p = 0.0001
	No	Reference	
	Yes	5.87 (2.47-13.93)	
	HIV Status	2.67 (0.85-8.42)	p = 0.084
	Prev. genital infection		p = 0.026
	No	Reference	
	Yes	0.47 (0.23-0.95)	
	Pap smear history		p = 0.003
	Regular	Reference	
	Irregular	4.26 (1.20-15.11)	p = 0.025
	Never	6.98 (2.05-23.75)	p = 0.002
	CIN2 or above		p = 0.001
	No	Reference	
	Yes	3.44 (1.62-7.31)	
	CIN3 or above		p = 0.055
	No	Reference	
	Yes	2.14 (0.98-4.64)	
High-grade (≥ CIN2) histology:	HIV status	5.11 (1.02-26.23)	p = 0.039
	Smoking history	1.73 (0.92-3.24)	p = 0.059
	Age at 1st intercourse	1.15 (1.01-1.32)	p = 0.043
	Genital warts		p = 0.011
	Yes	Reference	
	No	1.37 (1.09-1.72)	
	HSIL in Pap test		p = 0.0001
	No	Reference	
	Yes	18.18 (6.48-51.01)	
	Colposcopy ATZ2		p = 0.001
	No	Reference	
	Yes	3.44 (1.62-7.31)	

* Binary logistic regression analysis with backward stepwise LR statistics; Crude OR (95% CI).

Discussion

During the past several years, our multi-centre study has produced new data on the prevalence, risk factors, detection and behaviour of HPV infections and CIN in HIV-infected women in Italy [8-11, 13, 25, 26]. The DIANAIDS project was recently concluded by an analysis of the factors predicting the persistence and clearance of HPV infections and Pap smear abnormalities in these women during a prospective follow-up [11]. These data suggest that HIV-infected women even on HAART demonstrate a more aggressive clinical course of cervical HPV infections, and fail to eradicate the disease more frequently than HIV-negative women. Because this persistence of HPV infection and Pap smear abnormality can be predicted by the Pap test and HPV typing, our data suggest that in addition to regular monitoring by Pap smear, HPV testing for the oncogenic HPV types seems to provide additional prognostic information in the management of cervical lesions in HIV-infected women [11].

In addition to the accumulated clinical data on HPV-associated cervical pathology in HIV-positive women [5-7, 12, 14-20], new interesting data from in vitro studies have been provided recently [34-38]. It seems obvious

Table 4. — *Significant independent predictors of intermediate endpoint markers in multivariate logistic regression analysis.*

Dependent variable	Covariates	Crude OR (95% CI)	*Adjusted OR (95% CI)	Significance
HSIL in Pap test:#	HIV Status	4.50 (1.08-18.85)	5.20 (1.16-23.16)	p = 0.031
	Pap history:			
	Regular	Reference	Reference	p = 0.047
	Irregular	3.58 (1.01-12.78)	5.27 (1.33-20.83)	
	Never	4.02 (1.05-15.37)	5.79 (1.28-26.13)	
ATZ2 colposcopy:#	HSIL in Pap			
	No	Reference	Reference	p = 0.0001
	Yes	5.87 (2.47-13.93)	6.28 (2.41-16.33)	
	Previous genital infection			p = 0.044
	No	Reference	Reference	
	Yes	0.47 (0.23-0.95)	0.42 (0.18-0.97)	
	Pap smear history			p = 0.040
	Regular	Reference	Reference	
	Irregular	4.26 (1.20-15.11)	1.82 (0.37-8.85)	p = 0.456
	Never	6.98 (2.05-23.75)	4.59 (1.01-20.89)	p = 0.050
High-grade (≥ CIN2) histology:#	HSIL in Pap test			
	No	Reference	Reference	p = 0.0001
	Yes	18.18 (6.48-51.01)	22.78 (4.43-117.07)	
	Colposcopy ATZ2			
	No	Reference	Reference	p = 0.008
	Yes	3.44 (1.62-7.31)	5.18 (1.54-17.39)	

*Adjusted for the other variables tested in the model; NC, not computable; ATZ2, atypical transformation zone grade 2; #only the factors in the causal pathway are included

that HIV can modulate HPV gene expression by different mechanisms, which, however, remain largely obscure at the moment. Unfortunately, our DIANAIDS project suffered from the lack of availability of histological biopsies for detailed analyses of molecular markers, thus we were unable to make an in-depth penetration into the mechanisms of HPV-HIV interactions [8-11, 13]. In our new HPV-PathogenISS study, a prospective cohort is combined with a retrospective series of biopsies from precancerous lesions and cancer, to be analysed with a wide spectrum of molecular markers targeting the key molecular pathways [39]. The rationale behind this approach is to first assess the predictive value of this panel of markers in a retrospective series and subsequently apply the most informative markers in the analysis of the lesions from the prospective cohort. This is necessary to shed more light on the mechanisms by which these two viruses contribute to the more aggressive behaviour of CIN lesions in HIV-infected women, even on HAART [5-7, 11, 12, 14-20].

Three plausible explanations might be offered to explain this aggressive behaviour of CIN in HIV-positive women: 1) Firstly, the exposure level of these women to the known risk factors (most notably HPV) of cervical cancer might be different from that of HIV-negative women, 2) HIV-induced immunosuppression results in failure of the immunological mechanisms to eradicate oncogenic HPV infections [11], or 3) direct molecular interactions between HPV and HIV [34-38]. Self-evidently, these are not mutually exclusive, but more likely, are interrelated, and might act even synergistically to each other [1,2]. Whereas the molecular interactions remain largely unexplored, there is no doubt that HIV-associated immunosuppression is of major importance as a risk factor of HPV infections [1, 2, 46]. The first of the three options, i.e., different level of exposure to the known risk factors of cervical cancer is not fully explored as yet.

Included in our study protocol is a detailed questionnaire recording the data on exposure of the women to the known risk factors of CIN, HPV and cervical cancer [1, 2]. The analyses of these data indicate that the exposure of HIV-positive and HIV-negative women to the key risk factors is significantly different (Table 1). Accordingly, the number of life-time sexual partners is significantly ($p = 0.0001$) higher in HIV+ women as is the age at onset of sexual activity ($p = 0.002$) and contraception (yes-no) ($p = 0.009$). Several other factors, e.g. smoking habits discriminate these two groups of women. These data clearly substantiate the view that these two groups of women are significantly different in their status of exposure to the key risk factors of cervical cancer, most notably those related to sexual behaviour [1, 2].

We next analysed whether this different exposure status is directly related to differences in the clinical status of the women (Table 2). The differences between the two groups were much less marked as could be anticipated on the basis of the data in Table 1. Due to the small number of HIV+ cases, however, many of these observed differences reached a borderline statistical significance only. Most notably, however, HIV+ women had a higher frequency of HSIL in the Pap test, and high-grade CIN, both reaching statistical significance at the 5% level ($p = 0.05$). Albeit our HPV detection data are still very much premature, HPV prevalence (particularly with SPF primers) seems to be higher in HIV+ women, which is

consonant with the data generally reported in HIV patients [6-21].

HSIL in Pap test and high-grade CIN in biopsy are intermediate endpoint markers in the causal pathway towards cervical cancer [1-3]. Thus, a higher frequency of these intermediate endpoints would be consistent with an increased risk of developing cervical cancer. Indeed, this seems to be the case in HIV+ women, who show both HSIL and high-grade CIN more frequently than their HIV- counterparts. Another such intermediate endpoint marker is high-grade abnormality in colposcopy, i.e., ATZ2 [41, 42]. The next logical step was to analyse whether the occurrence of these intermediate endpoint markers could be predicted by a) the exposure status to the risk factors of cervical cancer (as recorded by questionnaire), or b) any of the clinical examinations done for these women. In univariate analysis (Table 3), HSIL in the Pap test was predicted by the HIV status, age at first sexual intercourse, and Pap smear history (particularly never having taken a Pap test). The predictive value of all these variables was not very impressive, however. In contrast, high-grade abnormality in colposcopy (ATZ2) was predicted significantly by the HSIL in the Pap test ($p = 0.0001$) and Pap smear history ($p = 0.003$). Interestingly, HIV status was not a significant predictor of ATZ2 colposcopy.

Most importantly, however, a biopsy-confirmed high-grade lesion (CIN2 or higher) was significantly predicted by several variables, most notably by HSIL in the Pap test ($p = 0.0001$) and ATZ2 colposcopy ($p = 0.001$). HIV status and age at first sexual intercourse were also predictors, but with much less statistical power. Interestingly, high-grade CIN was negatively predicted by the detection of external genital warts, i.e., was less common in these cases. This could suggest that women with external genital warts, which are practically always caused by the low-risk HPV types 6 and HPV11, might be at lower risk of contracting an infection by the high-risk HPV types and thus at lower risk for developing high-grade CIN. This remains to be confirmed by the HPV detection data, however. Importantly, age was not a predictive factor of high-grade CIN, and thus unlikely to be a confounding factor in multivariate analysis.

When all the factors significant in univariate analysis were entered in the multivariate models (and controlled to exclude the possible confounding by age), the results are highly interesting (Table 4). Pap smear history retained its power as an independent predictor of HSIL and high-grade colposcopy, whereas HIV status only predicted HSIL, but lost its power to predict ATZ2 and high-grade CIN. By far the most significant predictor of ATZ2 was HSIL Pap smear, whereas high-grade CIN was significantly predicted only by two factors: HSIL Pap test and ATZ2 colposcopy. Interpretation of these data might offer new insights into several potential interactions of these two viruses in HPV-associated cervical pathology.

Following the regular sequence of diagnosis, an abnormal Pap test is followed by colposcopy and punch biopsy. High-grade lesions in any of these can be regarded as intermediate endpoint markers of cervical cancer. In fact, only one intermediate endpoint marker exists, a high-grade cancer precursor lesion, measured by these three diagnostic techniques and called by three different names: HSIL, ATZ2 and high-grade CIN. Ideally, these three techniques should be strongly concordant. As shown by the present data, this seems to be the case because each the two techniques is the most powerful predictor of the third (Table 3 and 4).

However, there seems to be significant differences between the three as to their other potential predictors. Thus, only HSIL Pap test and ATZ2 are independent predictors of biopsy-confirmed high-grade CIN, which can be regarded as the most accurate of these intermediate endpoint markers. Importantly, HIV status was not an independent predictor of high-grade CIN in this study, but only predicted HSIL in the Pap test. This indicates that not all of these independent predictors of the HSIL Pap test and ATZ2 are equally accurate predictors of the "true" intermediate endpoint marker of cervical cancer, i.e., high-grade intraepithelial lesion confirmed in biopsy. The most plausible explanation is that some of the HSIL in the Pap test do not represent a histological high-grade lesion, and the same is true with some of the high-grade abnormalities on colposcopy. In the case of a HSIL smear, the cause may be a) misclassification, or b) regression of the Pap smear abnormality before confirmed in the biopsy [2]. Concerning colposcopy, this technique suffers from inherent low specificity, which explains that some of the ATZ2 lesions are not related to true cancer precursors [1, 2, 25, 42]. Thus, although strongly interrelated, the HSIL Pap smear, ATZ2 colposcopy and high-grade CIN are not equivalent intermediate endpoint markers of cervical cancer, and of these, biopsy confirmed CIN should be used as the "gold standard". It is important to emphasize, however, that some of the CIN3 lesions will eventually regress without ever progressing to invasive disease, making even this an "imperfect" intermediate endpoint marker of cervical cancer [1-3].

Of the known risk factors of cervical cancer (Table 1), only few remained independent predictors of the three intermediate endpoint markers (Table 4). Thus, HIV exposure is a risk factor for developing HSIL in the Pap test and never having taken a Pap test were significant predictors of both HSIL Pap and ATZ2 colposcopy. Neither of these, however, predicted high-grade CIN, i.e., had an independent predictive power exceeding that of HSIL or ATZ2. This would suggest that exposure to HIV is not necessarily the key determinant of high-grade CIN, but is confounded by other more significant predictors. Most likely, the determinants of high-grade CIN and cervical cancer in these patients are highly complex, and reflect an interplay between HIV, HPV and other possible risk factors.

The present analysis suffers from the lack of HPV detection data and the limited number of HIV-positive cases. To fully explore these complex interactions, we need to await the enrolment of additional HIV-positive women. To disclose the eventual basic differences

between these two groups, these analyses need to be completed separately in HIV+ and HIV- women. Once available, these data should enable more accurate evaluation as whether the increased risk of HIV-positive women to develop high-grade CIN is due a) to their different exposure status to the risk factors (which seems obvious in the present series), b) to direct effects of HIV, or c) to unknown interactions of the two viruses, HIV and HPV, at a molecular level.

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