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Cytokines and chemokines levels in primary HPV infection: a pilot study

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Summary. – The purpose of the study was to compare cytokines (CK) and chemokines concentrations in blood and cervico-vaginal samples between human papillomavirus (HPV)-positive and HPV-negative women, who had no previous history of HPV infection. A case-control study compares the activity and the concentration of CK/chemokines between 19 HPV-positive and 22 HPV-negative women matched by age. Plasma and cervico-vaginal levels of CK and chemokines were measured using cytofluorimetric analysis and expressed as mean of percentages. Plasma rates of interleukin (IL)-6 were significantly greater in HPV-negative women (mean value of 5.20±4.79 pg/ml) in comparison with HPV-positive women (mean value of 2.57±3.09 pg/ml) (p = 0.001). On the contrary, plasma levels of Eotaxin and hMCP-1 were significantly higher in HPV-positive women, with a mean value of 13.87±4.54 pg/ml (p = 0.022) and 53.53±19.51 pg/ml (p = 0.005), respectively. Differences in cervico-vaginal CK/chemokines concentrations were statistically not significant. Difference in plasma concentrations of IL-6, Eotaxin, IL-1β and hMCP-1 was statistically significant even by analyzing HPV-16/18 and multiple HPV genotypes infections. Primary HPV infection shows a characteristic pattern of plasma CK/chemokines concentration as opposed to HPV-negative subjects and persistent HPV infection.

Keywords: chemokines; cytokines; HPV primary infection; plasma pattern

Introduction

The natural history of human Papillomavirus (HPV) infection reflects the host's immune response: viral clearance occurs after at least 2 years, while in 5–10% of cases virus persistence causes intraepithelial cervical cancer (CIN) and less frequently cervical cancer (Cromme *et al.,* 1993*;* Moscicki *et al.,* 2000; Nobbenhius *et al.,* 2000; Munoz *et al.,* 2003).

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Abbreviations: CIN = cervical intraepithelial neoplasia; CK = cytokine; HPV = human papillomavirus; IL = interleukin; IP = induced protein; MCP = monocyte chemoattractant protein; n.d. = not detectable; PE = Phycoerythrin; TNF-α = tumor necrosis factor α; T-reg = regulatory T cells

The first host response depends on the tissue-innate immune system such as mucosal lactoferrin (LF) and myeloperoxidase (MPO), and it contributes to an apoptosis-induced intercellular pathway of malignant cells (Song and Santanam, 2001; Mistry *et al.,*2007; Gardella *et al.,* 2016).

Recent studies demonstrated that the cell-mediated immune response (T-helper cells and cytotoxic lymphocytes) triggers the production of a cascade of cytokines (CK) and it determines the progression of HPV infection, both systemically and locally (Wu and Kurman, 1997). CK are classified as Th1-type (tumor suppressing) such as interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 2 (IL-2) and 12 (IL-12), which elicit a cell-mediated immune response, and Th2-type, including IL-4, IL-5, IL-6, IL-8 and IL-10, which, on the other hand, inhibit the cell-mediated immune response (Clerici *et al.,* 1994)

In the past, few studies have shown a shift of the immune response from Th1 to Th2 in patients with HPV-associated CIN (Zhang *et al.*, 2011). HPV infection has been reported to inhibit the activation of natural killer cells, with an imbalance in the ratio of Th1 and Th2, resulting in Th2 overexpression and subsequent local immunosuppression (Shi *et al.*, 2013).

The aim of this study was to compare CK and chemokines concentrations between HPV-positive and HPV-negative women, who had no previous history of HPV infection. In addition, the pattern and the concentration of inflammatory CK and chemokines expressed in the blood stream and in the mucosal tissue of subjects affected by primary HPV infection were evaluated.

Materials and Methods

Patient enrollement and sample collection. A case-control study was performed comparing the activity and the concentration of CK/chemokines between HPV-positive and HPV-negative women matched for age. The Institutional Review Board of the Hospital approved the study and informed consent was obtained from all subjects. Women aged between 18 and 69 years attending the Colposcopy Clinic of the Department of Obstetrics and Gynecology of the IRCCS Fondazione Policlinico San Matteo, from July 2015 to February 2016 because of an abnormal Pap-smear, were enrolled as cases. Volunteer healthy subjects aged between 18 and 69 years, during routinely visit, were enrolled as controls. Patients of both groups should have had no previous history of either HPV infection or other gynecological disease. Exclusion criteria included pregnancy, congenital or acquired immunodeficiency syndromes, previous or concomitant genital neoplasia, previous total hysterectomy, use of non-steroidal anti-inflammatory drugs and/or use of vaginal medication in the previous week. All patients were treated according to an established protocol, including HPV-DNA detection and typing by using the line probe assay INNO-LiPA HPV genotyping assay (Innogenetics N.V., Ghent, Belgium), which previously demonstrated a clinical sensitivity of 95% in the identification of high-grade SIL and proved to be highly suitable for clinical and epidemiological investigations (Safaeian *et al.*, 2007). After speculum examination, two consecutive lavages after the administration of 10 ml sterile salt water solution were performed, followed by aspiration of the suspension after one minute of pooling and retention in sterile phials. Thereafter, patients underwent colposcopy with targeted biopsies in the event of abnormal Pap-smear and/or colposcopic findings. All exams were performed by two expert gynecology residents certified by the Italian Society of Colposcopy and described according to the 2011 Colposcopic Terminology of the International Federation for Cervical Pathology and Colposcopy (Boernstein *et al.*, 2012). Peripheral blood was obtained from all enrolled patients through venipuncture into heparinized tubes (BD Bioscience, Buccinasco, Italy). All patients enrolled as controls underwent another colposcopy one year after, while HPV-positive women were treated according to the 2015 guidelines of the Italian Society of Colposcopy.

Cytofluorimetric analysis. Levels of TNF-α, IL-1β, IL-1RA, IL-8, IL-12p70, IL-6, IL-10 (Human inflammation 7-plex) and Eotaxin, IP-10, CXCL8, MCP-1, MCP-3, RANTES (Human Chemokine 6-Plex) were measured on vaginal lavages and peripheral blood samples of each subject, by a multi-parameter sandwich ELISA assay (AimPlexTM) based on the use of microsphere technology, and flow cytometry analysis, which analyzes multiple analytes simultaneously in a single reaction. The staining procedure requires 60 min of sample incubation with the capture antibodies bound to the microspheres, 30 min incubation with specific detection antibodies, and lastly, 20 min of incubation with Streptavidin conjugated to Phycoerythrin (PE). The analyte is detected and quantified by the use of a biotinylated antibody. This antibody recognizes an epitope of the target protein that is different from the one recognized by the capture antibody. A treatment with Streptavidin conjugated to PE follows this procedure. The fluorescence intensity of PE detected in the second channel fluorescence (FL-2) is directly proportional to the amount of the target protein in the sample. The instrument used to study multiple profiles of CK/chemokines and detect microspheres is a CyFlow Space Partec equipped with a 635 nm laser and a 488 nm laser, which serves as a reporter for the quantification of the PE signal. The protein concentrations in the samples, expressed as pg/ml, are obtained using the fluorescence signal MFI (Mean Fluorescence Intensity). MFI values were converted into concentrations by Flowcytomix Pro software, version 2.3.

Statistical analysis. Patients' characteristics and plasma and cervico-vaginal CK/chemokines concentrations of cases and controls were summarized and tabulated by count, mean, standard deviation (SD) or percentage as appropriate. Continuous data were compared by Mann-Whitney U test. P-value < 0.05 was considered statistically significant. Data were analyzed with Stata/MP 10 for Windows (StataCorp LP, College Station, TX, USA).

Results

Forty-one women were enrolled in the protocol study, but 3 patients were excluded due to pregnancy. Finally, 19 cases and 19 controls were analyzed. At enrolment, 31.6% (6/19) of cases and 73.7% (14/19) of controls were nulliparous, while 68.4% (13/19) and 26.6 % (5/19), respectively, had delivered at least once. The 94.7% (18/19) of the case group and 68.4% (13/19) of the control group were not using any form of contraception. In the meantime, 26.3% (5/19) of controls and 5.3% (1/19) of cases were using estro-

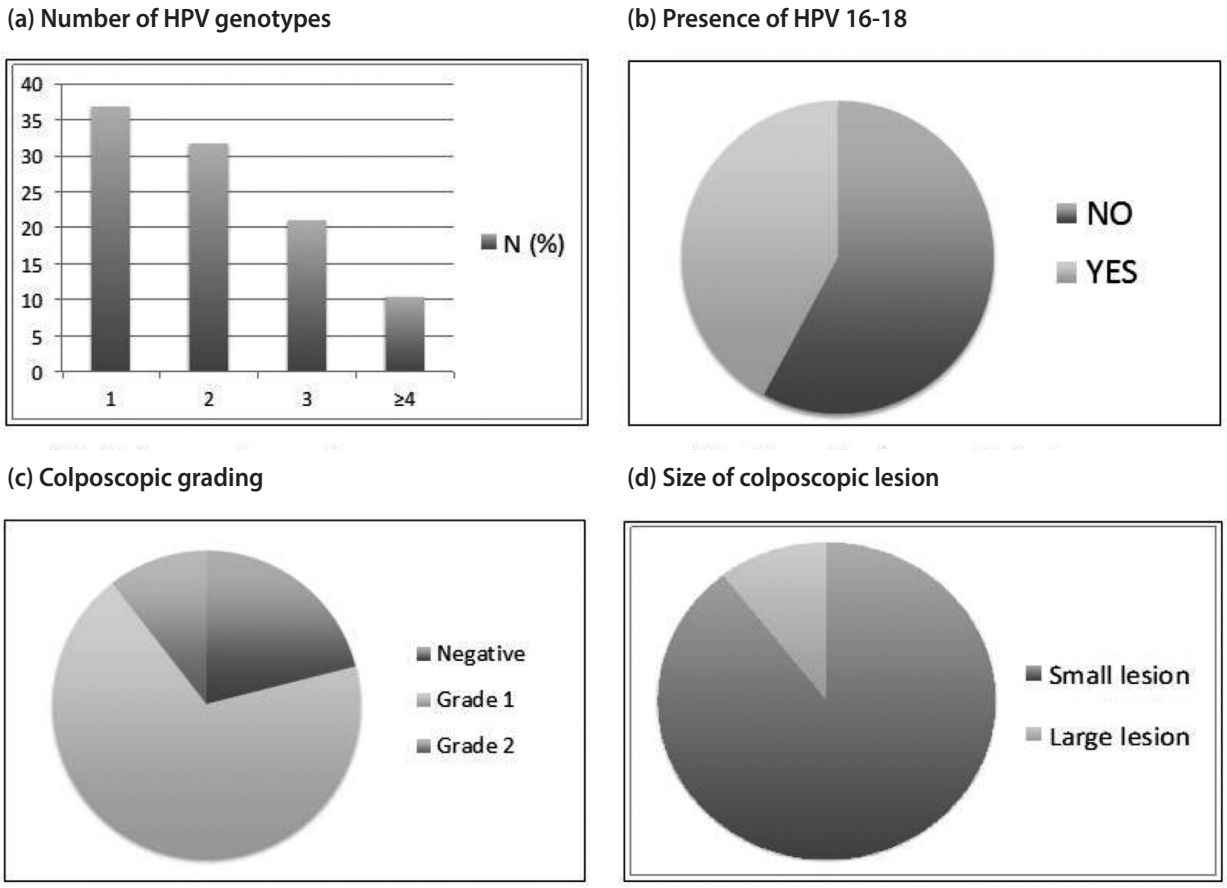

Fig. 1

HPV detection and colposcopic findings of cases (n = 19)
(a) number of HPV genotypes; **(b)** presence of HPV 16-18; **(c)** colopscopid findings; **(d)** size of colposcopic lesion.

Table 1. Plasma levels of cytokines and chemokines in HPV-positive (cases) and HPV-negative (controls) women

	Controls		Cases		p-value
	Mean	SD	Mean	SD	
IL-1RA	7.14	8.83	8.50	9.95	0.665
IL-8	266.45	446.41	142.26	229.90	0.297
IL-12p70	1.07	4.49	1.11	4.47	0.817
IL-6	5.20	4.79	2.57	3.09	0.001
IL-10	1.15	1.71	1.17	1.55	0.908
TNF-alfa	1.16	2.64	1.65	3.50	0.931
IL-1beta	2.64	2.89	1.64	3.33	0.138
Eotaxin	13.87	4.54	21.04	9.52	0.022
hIP-10	9.82	9.93	12.07	11.95	0.729
hIL- 8	142.64	157.18	115.98	220.01	0.525
hMCP-1	43.37	43.63	53.53	19.51	0.005
hMCP-3	7.51	5.32	11.17	7.40	0.201
hRANTES	4548.42	1507.70	4989.68	890.16	0.201

Legend: SD, standard deviation; IL, interleukin; TNF, tumor necrosis factor; IP, induced protein; MCP, monocyte chemoattractant protein.

Table 2. Cervico-vaginal levels of cytokines and chemokines in HPV-positive (cases) and HPV-negative (controls) women

	Controls		Cases		p-value
	Mean	SD	Mean	SD	
IL-1RA	844.18	1587.73	521.17	872.43	0.885
IL- 8	968.65	2152.36	464.89	752.31	0.583
IL-12p70	2.55	4.64	1.96	2.54	0.817
IL-6	27.56	67.44	8.75	8.05	0.525
IL-10	5.53	2.97	6.54	2.80	0.212
TNF-α	2.58	3.08	2.53	1.11	0.339
IL-1β	47.29	152.33	11.10	16.64	0.708
Eotaxin	n.d.	n.d.	n.d.	n.d.	1.000
hIP-10	0.90	2.30	2.38	5.42	0.402
hIL-8	561.65	1389.63	287.50	499.89	0.488
hMCP-1	9.36	6.74	12.28	8.99	0.154
hMCP-3	4.33	3.44	5.93	4.46	0.271
h-RANTES	14.93	20.17	10.15	16.86	0.271

Legend: SD, standard deviation; IL, interleukin; TNF, tumor necrosis factor; n.d., not detectable; IP, induced protein; MCP, monocyte chemoattractant protein.

progestins. Only 5.3% (1/19) of controls were using condom, while none of cases were using this barrier method. 42.1% (8/19) of controls and 26.3% (5/19) were smokers. All patients with a negative Pap-smear were HPV-negative and had normal colposcopic examination. HPV-16 and HPV-18 were detected in 41.1% of cases. Multiple genotypes were found in 63% of HPV-infected patients (Fig. 1a,b). Colposcopic findings are summarized in Fig. 1c,d.

After one year, all controls had negative colposcopic examination. In contrast, among the 17 remaining cases (2 patients were lost at follow-up), 1 underwent loop electrosurgical excision procedure (LEEP) for CIN3, 6 had a negative Pap-smear but still showed grade 1 abnormal findings at colposcopy and 10 had cytological and colposcopic features of CIN1. Plasma concentrations of CK and chemokines are reported in Table 1.

Plasma levels of IL-6 were significantly higher in HPV-negative women, showing a mean value of 5.20 ± 4.79 pg/ml (range: 2.6–24 pg/ml) in controls versus 2.57 ± 3.09 pg/ml (range: 0–13.37 pg/ml) in cases (p = 0.001). On the contrary, plasma levels of Eotaxin were significantly higher in HPV-positive women, with a mean value of 13.87 ± 4.54 pg/ml (range: 7.68–25.52 pg/ml) in the control-group and 21.04 ± 9.52 pg/ml (range: 10.72–38.72 pg/ml) in the case-group (p = 0.022). Also, plasma concentration of hMCP-1 showed a statistically significant difference between HPV-negative (43.37 ± 43.63 pg/ml, range: 17.35–217.29 pg/ml) and HPV-positive patients (53.53 ± 19.51 pg/ml, range: 22.77–83.06 pg/ml) (p = 0.005). No statistical difference was proven in plasma concentration of all other CK/chemokines. Cervico-vaginal CK/chemokines concentrations in controls and cases are reported in Table 2. Differences in none of these were statistically significant.

Table 3 shows comparison of plasma levels of CK/chemokines between cases and controls, by considering only cases with HPV-16 and/or HPV-18 infection. Likewise, the mean plasma concentration of IL-6 was significantly greater in HPV-negative women (4.56 ± 4.40 pg/ml, range: 0–24) (p = 0.007), whereas the mean plasma level of Eotaxin was significantly higher in HPV-16/18 positive women (29.22 ± 8.48 pg/ml, range: 12.10–38.72) (p <0.001). Also, plasma rates of IL-1β showed a statistically significant difference between HPV-negative and HPV-16/18 positive patients, with mean values of 2.71 ± 3.29 pg/ml (range: 0–12.51) and 0.00 ± 0.00 pg/ml (range: 0.00–0.00), respectively (p = 0.021). Furthermore, difference in plasma concentrations of IL-6, Eotaxin and hMCP-1 was found to be statistically significant even by comparing multiple and single HPV genotypes infections. In fact, plasma IL-6 showed a mean value of 1.81 pg/ml (range: 0.00–3.45 pg/ml) in patients with single HPV infection and 3.02 pg/ml (range: 0.00–13.37 pg/ml) in patients with multiple HPV infections (p = 0.006). Mean plasma concentration of Eotaxin was 17.76 pg/ml (range: 10.81–37.84 pg/ml) in patients with single HPV infection and 22.96 pg/ml (range: 10.72–38.72 pg/ml) in patients with multiple HPV infections, respectively (p = 0.031). Mean plasma level of hMCP-1 was 52.38 pg/ml (range: 22.77–83.06 pg/ml) and 54.21 pg/ml (range: 22.88–80.40 pg/ml) in patients infected by single and multiple HPV genotypes, respectively (p = 0.022). No

Table 3. Plasma levels of cytokines and chemokines in HPV-16/18 positive (cases) and HPV-negative (controls) women

	Controls		Cases		p-value
	Mean	SD	Mean	SD	
IL-1RA	8.58	8.17	12.99	12.99	0.140
IL-8	232.71	393.16	98.04	112.04	0.661
IL-12p70	1.38	4.95	n.d.	n.d.	0.492
IL-6	4.56	4.40	1.38	1.97	0.007
IL-10	1.25	1.62	0.80	1.62	0.449
TNF-α	1.67	3.36	0.42	1.19	0.538
IL-1β	2.71	3.29	n.d.	n.d.	0.021
Eotaxin	14.32	4.49	29.22	8.48	<0.001
hIP-10	10.55	10.56	12.44	12.77	0.765
hIL-8	145.28	209.38	69.42	46.34	0.635
hMCP-1	46.81	37.16	54.62	15.46	0.082
hMCP-3	9.05	6.19	10.41	8.44	0.765
hRANTES	4711.08	1257.76	4986.4	1234.82	0.986

Legend: SD, standard deviation; IL, interleukin; n.d., not detectable; TNF, tumor necrosis factor; IP, induced protein; MCP, monocyte chemoattractant protein.

statistically significant difference in plasma and cervico-vaginal CK/chemokines was proven in smokers when confronted to non-smokers.

Discussion

Recent studies confirmed that the innate and the adaptative cell-mediated immunity play a key role in promoting regression and in preventing recurrences of HPV infection (Wu and Kurman, 1997; Bais *et al.,* 2005). Analysis of plasmatic samples has demonstrated a shift from the Th1 to the Th2 response in patients with HPV-associated dysplasia (Zhang *et al.,* 2011). In particular, IL-12 promotes the Th1 response, which is crucial in determining the virus clearance via releasing TNF-α, INF-γ and IL-2. In contrast, IL-10 promotes the Th2 response, which inhibits the cell-mediated immunity and induces the humoral immunity, releasing IL-4, IL-5, IL-6 and IL-8 (Zhang *et al*., 2011).

In a case-control study, Kemp *et al*. (2010) have evaluated the plasma concentration of few pro-inflammatory CK in a group of patients with persistent HPV infection and levels of IL-6, IL-8, TNF-α and MIP-α were significantly increased. In fact, high plasma CK concentrations are associated with a reduction of the antigen-specific response, resulting in persistency of HPV infection (Kemp *et al*., 2010).

Peghini *et al*. (2012) showed that high levels of Th1 cytokines could be detected in 75% of patients with negative pap-smear and only in 10% of patients affected by HPV-related cervical lesions. In particular, the patients with invasive cervical carcinoma were characterized by a characteristic plasmatic called T-reg profile. T-reg cells (regulatory T cells) are considered to be crucial in determining peripheral tolerance and, thus, preventing autoimmune diseases. However, T-reg cells are also immunosuppressive by producing IL-10, IL-4, TGF-β1 and β2. High levels of TGF-β were detected in the case of progression to high-grade cervical lesions (Peghini *et al*., 2012).

Therefore, this can be considered as an original analysis that compares pattern of plasma and cervico-vaginal CK and chemokines between patients with HPV primary infection and HPV-negative women in order to clarify the exact mechanism of the cell-mediated response to HPV primary infection. Immunological tolerance is required in order to establish the persistence of HPV infection. Since HPV infection causes low systemic impact, various studies have been conducted to evaluate vaginal CK and chemokines levels in the past. Marks and al. studied the vaginal CK and chemokines profile in a group of patients with persistent HPV infection and observed high concentrations of non-specific innate immune CK. In particular, they evaluated the shift from IL-2 to Eotaxin. Eotaxin is a chemokine responsible for the recruitment and regulation of eosinophils and basophils. For this reason, eosinophils have been investigated as a potential prognostic factor of tumors of oral cavity (Marks *et al.,* 2011). As opposed to Marks' results, we did not find any significant difference in cervico-vaginal CK and chemokines rates between HPV-

positive and negative patients. However, we were able to show high levels of Eotaxin in the plasma of patients with HPV primary infection compared to HPV-negative women and this difference reached statistical significance. This result was confirmed in patients affected by HPV-16/18 and multiple HPV genotypes infections. The same results were not found in cervico-vaginal samples, probably due to small size of the population in the study.

Several authors proved high levels of IL-6 in the blood of patients with HPV-determined cervical lesions, supporting a Th2-mediated response (Giannini *et al.,* 1998; Taveraes-Murta *et al.,* 2008). However, this study showed much lower rates of IL-6 in HPV-positive patients, especially in subjects affected by HP-16/18 and multiple genotypes infections. These results could be explained by the characteristics of the population. In fact, the case-group is composed of patients with recent and primary HPV infection, showing low-grade cervical lesion in the majority of cases. In contrast, previous studies were conducted in women with mainly high-grade cervical lesions. The shift from Th1 to Th2 immune response probably requires more time; hence it could be easily identified in patients with persistent HPV infection and subsequent high-grade dysplasia.

MCP-1 has also been investigated as a potential marker of HPV-linked carcinogenesis. *In vitro* experiments conducted by Kleine-Lowinski et al. found a diminishing trend of plasma levels of MCP-1 during the progression of CIN to cervical carcinoma, in which this chemokine was not detected anymore (Kleine-Lowinski *et al.,* 1999). They also observed that high-risk HPV genotypes selectively suppressed MCP-1 as opposed to other chemokines. This chemokine pattern could probably be related to a major risk of progression to cervical cancer (Kleine-Lowinski *et al.,* 2003). This study, we identified statistically significant higher plasma concentrations of MCP-1 in HPV-positive patients and in particular in case of multiple HPV infection. This confirms the role of the monocyte-macrophage lineage as an important immunomodulator in primary HPV infection. High levels of MCP-1 in the group of patients could be interpreted as an overexpression of this chemokine during primary viral infection, which is followed by suppression if a progression to high-grade cervical dysplasia occurs. IL-1β is another CK known to regulate tumor progression via expression of growth factors, metastatic and angiogenic genes (Lewis *et al.,* 2006).

Behbakht *et al.* (2002) identified elevated vaginal IL-1β in patients with cervical dysplasia. We did not obtain the same results, but this could be caused by the lack of a standardized method of CK and chemokine analysis. On the other hand, these results show significantly higher plasma concentration of IL-1β in HPV-16/18 positive patients, underlying major risk for developing high-grade cervical dysplasia in the presence of elevated IL-1β levels.

The main limit of this study is the small size of the population under consideration and the lack of a standardized method to measure CK and chemokines in plasma and in cervico-vaginal secretions. A potential bias is a non-equal distribution of women using oral contraceptives (26.3% of controls versus 5.3% of cases) and with smoking attitudes (42.1% of controls versus 26.3% of cases). In fact, smoking has been recognized as an independent predictor of CIN in women with newly diagnosed HPV infection (Bairati *et al.,* 1999). Another possible confounder factor is the presence of cervico-vaginal infections: local infections may elicit immunologic and inflammatory responses of vaginal mucosa with a subsequent increased production of CK and chemokines, which could influence local viral replication (Santoni *et al.,* 2002).

According to our initial results, a primary HPV infection is not characterized by a Th2-mediated response, but by overexpression of chemokines responsible for recruitment of monocyte-macrophage lineage. A shift from Th1 to Th2 immune response could be involved when HPV infection becomes persistent and progression to high-grade cervical dysplasia occurs. However, further studies are necessary to evaluate local immune factors in primary and persistent HPV infections that could improve prediction, follow-up and management of early cervical dysplasia.

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