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Immunosuppression in cervical cancer with special reference to arginase activity

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HIGHLIGHTS

- Women with cervical cancer had higher levels of circulating T-helper-2-cell cytokines and arginase activity.
- Women with cervical cancer had lower levels of L-Arg and higher levels of L-Arg metabolites.
- High levels of IL-10 correlated with high levels of arginase activity in cervical cancer.

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ABSTRACT

Introduction. Cervical cancer is characterized by an immunosuppressive microenvironment and a Th2-type cytokine profile. Expression of arginase (ASE), the enzyme that converts L-arginine into L-ornithine and urea, is stimulated by Th2-type cytokines.

Objective. To assess the association of ASE activity and L-Arg metabolism products with cervical cancer.

Methods. Sera of 87 and 41 women with histologically confirmed by colposcopy-directed biopsy SCC and CIN3 respectively and 79 with normal cytology or Low-Grade Squamous Intraepithelial Lesion (LSIL), were evaluated. Cytokines were measured using Milliplex Human cytokine/chemokine kit. Arginase (ASE) activity was determined using an enzymatic assay. Levels of L-arginine, L-ornithine, putrescine and spermine were determined by HPLC.

Results. Significantly higher levels of ASE activity were observed in women with CIN3 (age-adjusted OR: 24.3; 95%CI: 3.82–155) and SCC (AOR: 9.8; 95%CI: 2.34–40.8). As expected, possibly due to high levels of ASE activity, higher levels of L-Arg were negatively associated with CIN3 (AOR: 0.03; 95%CI: 0.004–0.19) and SSC (AOR: 0.06; 95%CI: 0.02–0.24). Consistent with the role of ASE in the conversion of L-arginine to L-ornithine and polyamine production therefrom, women with cervical cancer had higher levels of spermine and putrescine. A correlation analysis revealed a significant albeit weak relationship between high levels of IL-10 and high levels of ASE (Pearson r = 0.32, p-value = 0.003) in women with cervical cancer.

Conclusion. This study indicates that ASE activity and L-Arg degradation mechanisms of immunosuppression are present in cervical cancer. The results foster research in the design of possible strategies to inhibit ASE activity for therapy of cervical cancer.

Introduction

The majority of Human Papillomavirus (HPV) infections and most of their related clinical manifestations clear spontaneously within 1 to 2 years after virus acquisition, but the small proportion of women that develop persistent infection with high-risk genotypes (HR-HPV) have high risk for cervical cancer [1]. There are cofactors that seem to

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modulate the risk of cervical cancer in HPV positive women: environmental (hormonal contraceptive use and smoking), viral (infection by specific HPV types, HPV variants and viral load) and host-related (genetics, hormones and immune responses) [2].

Individuals with altered cellular immune responses (i.e. HIV or transplants) are at increased risk of HPV-associated ano-genital cancers suggesting that cellular immune responses are essential to control both HPV infection and cancer [3]. T helper-mediated antitumor and antiviral responses critically depend on a functional cytokine network [4]. It has been demonstrated that women with squamous cell carcinoma (SCC) present increased levels of circulating IL-10 and decreased levels of interferon-gamma (IFN-γ) when compared to women with low grade lesions [5]. These observations suggest that a switch from Th1 to a Th2 cytokine pattern is associated with cervical cancer progression [5]. This is further supported by the observation that low IFN-γ mRNA levels in cervical exfoliates are associated with high grade lesions [Cervical Neoplasia 2/3 (CIN2/3)] [6].

In addition, recent research has demonstrated the existence of a complex relationship between immunity, tumorigenesis and L-arginine (L-Arg) metabolism, where the enzymes arginase (ASE) and inducible nitric oxide synthase (NOS2) metabolize L-Arg to polyamines (putrescine, spermine and spermidine) and nitric oxide (NO) which are essential products for tumor growth and tumor cytolysis, respectively [7,8]. On the other hand, NOS2 and ASE expression is differentially regulated by Th1 and Th2 type-cytokines. NOS2 can be induced by IFN-γ whereas ASE can be induced by IL-4, IL-10 and IL-13. ASE and NOS2 compete for the same substrate L-Arg which is crucial for T-cell function and for the modulation of tumor growth in the microenvironment [9,10]. The mechanisms involved in the regulation of L-Arg metabolism are not well known. Even further, there have not been studies evaluating these mechanisms in the progression of cervical cancer.

Several cofactors associated with risk of HPV persistent infection and high-grade cervical lesions seem to be associated with levels of cytokine expression. It has been observed that adolescent and young adult women using hormonal contraceptives have higher levels of IFN-γ, IL-10, and IL-12 mRNA in cervical exfoliates and that cigarette smoking is also significantly associated with low mRNA expression of IL-10 and IL-4 [11,12]. Furthermore, tobacco products may affect cell-mediated immune response by reducing the number of Langerhans (LC) and T-cells in the cervical transformation zone [13,14]. Expression of IL-10 evaluated by immunohistochemistry is lower in women presenting CIN3 who smoke [15] or use oral contraceptives [16]. These observations may suggest that the immunosuppressive environment observed in cervical cancer may be the result of a complex interplay between cofactors, cytokine induction and the enzymes of the L-Arg metabolism.

In this study we evaluated the association of circulating levels of cytokines, ASE activity and ASE-L-Arg metabolism products with CIN3 or SCC and investigated whether there is a correlation between the cytokine levels and ASE activity. In an effort to understand how different cofactors can interplay and/or interfere with the host immune response in the progression from persistent HPV infection to cervical cancer, we also explored the possible interaction of cofactors such as smoking, sexual behavior and oral contraceptives to modulate the association of cervical cancer with cytokines and L-Arg products.

Materials and methods

Patients

Eighty-seven women with SCC and 41 women with CIN3 that were histologically confirmed by colposcopy-directed biopsy were recruited from 7 cancer centers (Hospital Universitario San Vicente Fundación, Instituto de Cancerologia Las Américas, Clínica Medellin, Centro Medico Congregación Mariana, Clinica Vida, Hospital Marco Fidel Suarez and Hospital La Maria) in Medellin, Colombia. Seventy-nine women with normal cytology, or Low-Grade Squamous Intraepithelial Lesion (LSIL), included as normal controls were recruited among attendees of screening services affiliated with the University of Antioquia and Clinica Las Americas. Women with mental disabilities, pregnancy, HIV positive status, conization, radiation, chemotherapy or radical hysterectomy were excluded from the study, which was approved by both the bio-ethics committees of the School of Medicine and the Sede de Investigación Universitaria (SIU) of the University of Antioquia. All women participating in the study, signed informed consent.

Sample collection

Ten milliliter of blood was obtained by venipuncture using a red top BD Vacutainer® (San Diego, California) from all subjects (before treatment for women with disease). After blood withdrawal, vacutainers were immediately transported to the laboratory and allowed to clot for 30 min. After centrifugation at 3500 rpm for 10 min at room temperature, the serum was collected, aliquoted and stored at −80 °C until use. Biopsies were obtained by gynecologists at the time of surgical procedure and transported to the laboratory in 5 mL of RPMI-1640. In the laboratory, the biopsies were washed with PBS, weighed and frozen at −30 °C for subsequent DNA extraction and HPV detection. Cervical cells from women were collected during screening visit. After Pap smear preparation, the cytobrush was transferred to a tube with transport medium (Listerine, McNeli). In the lab, samples were vortexed, centrifuged and the cell pellet kept frozen at −30 °C until use.

Serum cytokine measurement

Sera from patients and normal controls were analyzed for IL1-β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IFN-γ, MCP-1, TNF-α and granulocyte macrophage colony-stimulating factor (GM-CSF) using the Milliplex MAP human cytokine/chemokine assay from Millipore (Catalog MPXHCYTO-60K, Millipore, Billerica, MA). VEGF and TGF-β (Catalog TGFB-64K-01, Millipore, Billerica, MA) were measured as a single analyte in a bead array according to the manufacturer's instructions. Beads were read on the Bio-Plex100 suspension array system and data were analyzed using Bio-plex Manager software, version 3.0 (Biorad, Hercules, CA).

Arginase activity

Serum samples were normalized to 25 μg of protein and were added to 25 μL of Tris–HCl (50 mmol/L; pH 7.5) containing $MnCl_2$. This mixture was heated at 55–60 °C for 10 min; then 150 μL of carbonate buffer (100 mmol/L; Sigma) and 50 μL of L-Arg (100 mmol/L; Sigma) were added, and the solution was incubated at 37 °C for 20 min. The reaction from L-Arg to L-ornithine was detected, at 515 nm, after addition of ninhydrin solution and incubation at 95 °C for 1 h.

Amino acid detection by HPLC

High performance liquid chromatography (HPLC) was conducted on deproteinized serum supernatants labeled with *O*-phtaldialdehyde (OPA). Analytes were eluted with 100 mM sodium acetate buffer, pH 5.0, with a linear gradient consisting of methanol (80%) and acetonitrile (80%). The analytes in the sample were calculated on the basis of standard curves of known amounts.

DNA extraction and HPV testing

DNA from biopsies was extracted using the Wizard® Genomic DNA purification kit (A1120, Promega, Madison, WI, USA) following the manufacturer's instructions. DNA from cervical smear crude extract was obtained using proteinase K (10 mg/mL proteinase K in 50 mM TRIS, pH = 8.3) as described [17]. HPV DNA was detected by the general primer $GP5^+/6^+$-mediated PCR described by Van den Brule [18]. As

positive control we used DNA from SiHa and Hela cell lines and the quality of all DNA samples was checked by amplifying a fragment of the β-globin using primers BG03/BG05 [19]. All samples that tested negative for hybridization after the $GP5^+/GP6^+$-mediated PCR, were subjected to HPV 16 and HPV 18 genotype specific PCR as described [20]. The primers used in this procedure amplify a fragment of 96pb (HPV-16) and 115pb (HPV-18) of HPV E6 gene which were visualized in 2% agarose gels stained with ethidium bromide.

Statistical analysis

The characteristics (age, age at first intercourse, lifetime number of sexual partners, parity, cigarette use, contraceptive use and HPV infection) of women with and without cervical cancer were compared using chi-square test of deviance and age-adjusted odds ratios (AORs) with 95% confidence intervals. The median of cytokine levels (pg/mL), arginase activity (nmol), L-arginine (μM), L-ornithine (μM), putrescine (μM), spermine (μM) and citrulline (μM) in women with SCC, CIN3 and women without cancer were estimated. The Kruskal–Wallis test was used to compare the median between the groups. Dunn's posttest following a Kruskal–Wallis was used for multiple comparisons to determine where the differences among the means occurred. We also assessed the association of CIN3 and cervical cancer with high levels (upper tertile) of cytokines, and molecules involved in the ASE-L-Arg metabolic pathway adjusted by age and HPV status using logistic regression models. Finally, we conducted an interaction analysis in which the risk of cervical cancer associated with high levels (upper tertile) of cytokines or molecules involved in the L-Arg-ASE metabolic pathway stratified by exposure levels of each of the risk factors (cigarette use, contraceptive use, number of lifetime sexual partners and HPV) was estimated using as reference the lower levels (≤upper tertile) and absence of the risk factor. Logistic regression models adjusted by age and HPV status were used to estimate the chi-square test of deviance for significance of interactions. The statistical program R version 2.9 (R Development Core Team (2009): A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0) was used for analysis. Correlation between the level of cytokines, and molecules involved in the ASE-L-Arg metabolic pathway was determined using the Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Results

Characteristics of the subjects included in the study

Two hundred and seven women (79 controls, 41 CIN3 and 87 SCC) from Medellin, Colombia were included in the study. Table 1 shows the distribution of age and risk factors, of the studied population. The median age of women with SCC was 45 years (range 24–76 years), with CIN3, 36 years (range 19–68 years) and without cervical cancer, 45 years (range 19–79 years). Table 1 shows the association of cervical cancer with first sexual encounter at young age (AOR: 11.97; 95%CI: 2.04–70.29, trend p-value: 0.008), high number of lifetime sexual partners (AOR: 4.98; 95% CI: 1.14–21.76) and HPV infection (AOR: 171; 95%CI: 25.49–1151, CIN3 vs. controls, AOR: 181.44; 95%CI: 36.53–901.15, SCC vs controls). The use of contraceptives and cigarette smoking were not associated with cervical cancer.

Association of cytokine levels with cervical cancer

First, we confirmed the association of cervical cancer with those cytokines considered key regulators of the L-Arg metabolism. As previously described, there was a statistically significant decrease in levels of circulating IFN-γ, IL-12p70 (Fig. 1A Supplementary material) and IL-4 (Fig. 1B, supplementary material) in women with CIN3 and SCC as compared to normal controls (Kruskal–Wallis test, $p < 0.0001$,

Table 1
Multivariate analysis of selected socio-demographic risk factors for cervical cancer of women from Medellin—Colombia, 2007–2010.

Factor	Controls (N = 79)	CIN3 (N = 41)	SCC (N = 87)	CIN3 vs controls	SCC vs controls	SCC vs CIN3
	n	n	n	OR[a] (95% CI)	OR[a] (95% CI)	OR[a] (95% CI)
Age year, median (range)[b]	45 (19–79)	36 (19–68)	45 (24–76)			
Age at first intercourse (year)						
≥20	33	9	8	1	1	1
16–19	39	20	37	0.76 (0.13–4.64)	2.19(0.44–10.91)	**5.52 (1.33–23.01)**
≤15	7	7	29	2.98 (0.22–40.20)	5.72 (0.65–50.36)	**11.97(2.04–70.29)**
Trend p				0.587	0.113	**0.008**
Missing values	0	5	13			
Lifetime sexual partners						
1	44	14	15	1	1	1
≥2	35	23	60	0.78 (0.14–4.25)	**4.98 (1.14–21.76)**	**3.78 (1.20–11.86)**
Missing values	0	4	12			
Number of pregnancies						
0–1	23	12	14	1	1	1
2–3	26	15	23	4.95(0.67–36.81)	2.62 (0.47–14.68)	0.59 (0.15–2.30)
≥4	30	13	47	6.06 (0.59–62.11)	3.29 (0.52–20.88)	0.84 (0.20–3.49)
Trend p				0.182	0.220	0.862
Missing values	0	1	3			
Use of contraceptives						
Never	28	9	29	1	1	1
Sometimes	51	30	51	0.72(0.11–4.89)	1.01 (0.25–4.10)	0.54 (0.16–1.86)
Missing values	0	2	7			
Use of cigarette						
Never	41	20	39	1	1	1
Sometimes	37	18	45	1.75 (0.36–8.44)	2.41(0.59–9.81)	0.69 (0.24–2.03)
Missing values	1	3	3			
HPV infection						
Negative	67	3	6	1	1	1
Positive	11	31	77	**171 (25.49–1151)**	**181.44 (36.53–901.15)**	0.65 (0.09–4.82)
Missing values	1	7	4			

Bold numbers denote statistically significant values.
[a] OR adjusted by age, age at first intercourse, lifetime sexual partners, number of pregnancies, use of contraceptives, use of cigarette and HPV infection.
[b] Age compared using the Mann–Whitney test: p-value CIN3 vs controls = 0.009; p-value SCC vs controls = 0.594; p-value SCC vs CIN3 = 0.001.

Dunn's test $p < 0.001$, Controls vs. CIN3 and controls vs. SCC). In contrast, the levels of circulating IL-10 and IL-13 were significantly higher in women with SCC as compared to women without cancer or CIN3 (Kruskal–Wallis test, $p < 0.0001$, Dunn's test $p < 0.001$, controls vs. SSC and CIN3 vs. SCC, Fig. 1B, supplementary material). We also observed higher levels of circulating IL-12p70 and IL-4 in women with SCC as compared to CIN3 (Kruskal–Wallis test, $p < 0.0001$, Dunn's test, CIN3 vs. SCC, Fig. 1A, supplementary material).

In order to evaluate the role of other factors in this relationship, we conducted a logistic regression analysis adjusted by age and HPV status as shown in Table 2. This analysis shows a clear pattern of association of SCC with high levels of IL-10 (OR: 59.6; 95%CI: 7.18–495) and IL-13 (OR: 3.57; 95%CI: 1.08–11.8). In contrast high levels of Th1 type cytokines IFN-γ (OR: 0.01; 95%CI: 0.001–0.12) and IL-12p70 (OR: 0.01; 95%CI: 0.001–0.07) were negatively associated with SCC. We also evaluated the association of high cytokine levels with the probability of invasion by comparing SCC with CIN3 (Table 2) observing that Th2 type cytokines IL-10 (OR: 40.0; 95%CI: 8.07–198) and IL-13 (OR: 21.3; 95% CI: 2.77–164) were associated with SCC.

Association of arginase activity and products of the L-arginine metabolic pathway with cervical cancer

Th1 and the Th2 cytokines are main inducers of NOS2 and ASE respectively; therefore we enquired whether ASE activity as well various metabolic compounds that are the product of ASE enzymatic activity are associated with the risk of cervical cancer. Since spermine is a derivative of putrescine, which in turn is produced from L-ornithine by action of ODC (ornithine decarboxylase) we also enquired whether the levels of these products are associated with cervical cancer. Fig. 1 shows the comparison of levels of those metabolic compounds between the groups included in the study. In general sera of women from the control group exhibit levels in the ranges described in other populations for all metabolic compounds [21]. However the levels of all metabolic compounds of sera of SCC and CIN3 cases differed markedly and significantly ($p < 0.0001$, Kruskal–Wallis test) from controls. Specifically, sera from both SSC and CIN3 cases exhibited higher levels of ASE activity (Fig. 1A) and spermine (Fig. 1E) and lower levels of L-Arg (Fig. 1B). The levels of L-ornithine (Fig. 1C) were significantly lower in CIN3 but those observed in SCC were not substantially from controls and levels of putrescine (Fig. 1D) were significantly higher in SCC cases only.

Since these data suggest the involvement of the L-Arg metabolic pathway in the course of the disease, in an effort to investigate the independence of this association, we adjusted by age and HPV status using logistic regression. As shown in Table 3, high levels (upper tertile) of ASE activity were significantly associated with CIN3 (OR: 24.3; 95%CI: 3.82–155) and SCC (OR: 9.8; 95%CI: 2.34–40.8). As expected, possibly due to high levels of ASE activity, higher levels (upper tertile) of L-Arg were negatively associated with CIN3 (OR: 0.03; 95%CI: 0.004–0.19) and SSC (OR: 0.06; 95%CI: 0.02–0.24) meanwhile high levels of spermine were associated with CIN 3 (OR: 3042;95%CI: 133.95–69,082) and SCC (OR: 82.3; 95%CI: 4.92–1376). We also observed high levels of putrescine associated to SCC (OR: 149; 95%CI: 15.6–1420) and although, high levels of L-ornithine were not associated with either CIN3 or SCC, high levels of this metabolite were associated with SCC when compared to women with CIN3 (OR: 10.3; 95%CI: 2.21–48.1).

Relationship between cytokine levels and ASE-L-Arg metabolic pathway

Fig. 2 shows that high levels of IL-10 are correlated with high levels of ASE activity in SCC cases. Although this correlation is significant according to the Pearson test (p-value = 0.003), it was very weak (Pearson r = 0.32). The analysis did not show any other correlation between the level of cytokines, and molecules involved in the ASE-L-Arg metabolic pathway (data not shown).

Interaction between risk factors and cytokine levels

The combined effect of high cytokine levels and risk factors associated with cervical cancer risk is shown in Table 4. The likelihood of SCC showed to be higher among women who in addition to cigarette smoking, contraceptive use, 2 or more lifetime sexual partners and HPV infection, had higher levels of circulating IL-10. Particularly, compared to women with lower levels of IL-10 and only one lifetime sexual partner, women with both high levels of IL-10 and 2 or more lifetime sexual partners had higher risk of SCC and this interaction was statistically significant (interaction p-value: 0.024).

Discussion

It has been suggested that elimination of HPV infection and hence the disappearance of cervical lesions are due to an adequate immune response [22]. Cytokines mediate many of immune response functions [23–25]. It has been observed that the risk of SCC is associated with increased circulating levels of Th2-type cytokines whereas high levels of Th1-type cytokines are associated with lower risk of SCC and CIN3 [5,6,11,15,26,27]. Indeed in our study we found that high levels of IL-10 and IL-13 were associated with SCC and that high levels of IFN-γ and IL-12p70 were associated with a reduction in SCC and CIN3 even after adjustment for age and HPV status. High serum levels of IFN-γ

Table 2
Bivariate analysis of association of high cytokine levels with SCC and CIN3 in women from Medellin—Colombia 2007–2010. Bold numbers denote statistically significant values.

Cytokine levels (pg/mL)	Controls (N = 79)	CIN3 (N = 41)	SCC (N = 87)	CIN3 vs controls	SCC Vs controls	SCC Vs CIN3
	n (%)	n (%)	n (%)	OR[a] (95% CI)	OR[a] (95% CI)	OR[a] (95% CI)
IFN-γ						
Others (≤1.37)	23 (29.1)	38 (92.7)	75 (86.2)	1	1	1
Upper tertile (>1.37)	56 (70.9)	3 (7.3)	12 (13.8)	**0.003 (0.0001–0.06)**	**0.01 (0.001–0.12)**	4.15 (0.50–34.5)
IL12p70						
Others (≤0.86)	28 (35.4)	35 (85.4)	83 (95.4)	1	1	1
Upper tertile (>0.86)	51 (64.6)	6 (14.6)	4 (4.6)	**0.03(0.004–0.21)**	**0.01 (0.001–0.07)**	0.39(0.09–1.77)
IL-10						
Others (≤0.12)	71 (89.9)	38 (92.7)	30 (34.5)	1	1	1
Upper tertile (>0.12)	8 (10.1)	3 (7.32)	57 (65.5)	0.63(0.07–5.75)	**59.6(7.18–495)**	**40.0(8.07–198)**
IL-13						
Others (0)	68 (86.1)	38 (92.7)	49 (56.3)	1	1	1
Upper tertile (>0)	11 (13.9)	3 (7.3)	38 (43.7)	0.14(0.01–1.50)	**3.57(1.08–11.8)**	**21.3(2.77–164)**
IL-4						
Others (≤0.06)	20 (25.3)	38 (92.7)	79 (90.8)	1	1	1
Upper tertile (>0.06)	59 (74.7)	3 (7.3)	8 (9.2)	0.01 (0.0007–0.1)	0.02 (0.003–0.10)	3.19(0.38–27.0)

Bold numbers denote statistically significant values.
[a] Ajusted by age and HPV status.

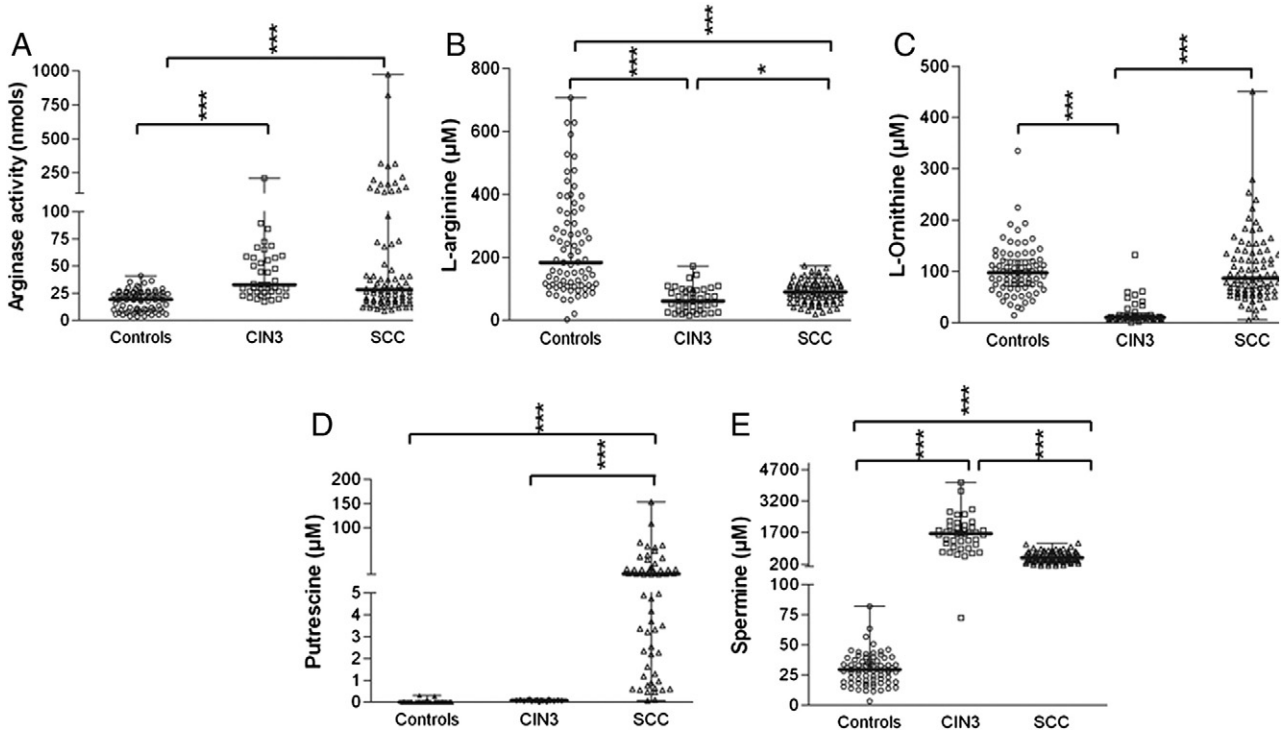

Fig. 1. Arginase activity and products of the L-arginine metabolic pathway in sera of women with and without cervical cancer. (A) Arginase activity levels (nmol), (B) L-arginine levels (μM), (C) L-ornithine levels (μM), (D) putrescine levels (μM) and (E) spermine levels. **p < 0.01 and ***p < 0.001, Dunn's test.

(>1.37 pg/mL) showed a very strong statistically significant negative association not only with SCC (Table 2, OR = 0.01; 95% CI = 0.001 to 0.12) but also with CIN3 (Table 2, OR = 0.003; 95% CI = 0.0001 to 0.06). This finding is further supported by the profile shown by IL-12p70 (>0.86 pg/mL) consistent with its role as promoter of Th1-type immune responses. Recently a cohort study evaluated the relationship between cytokine levels and clearance of 107 high-risk and 111 low-risk incident HPV infections. The authors found that high and low levels of IL-12 and IL-10 respectively were associated with HPV clearance [28]. It has been shown that HPV16 E6 and E7 proteins inhibit IFN-γ-mediated effector mechanisms [29]. Therefore, it is not surprising that in vivo studies show that levels of Th1-type cytokines are completely abolished in cervical cancer. On the other hand, HPV appears to have also the ability of inducing Th2-type cytokines since it has been observed that HPV 16 E6 protein stimulates IL-10 gene expression by modifying the response of its promoter [30]. In our study we observed that although SCC was associated with high levels of IL-10 as compared with women with no lesions (OR = 59.6;95% CI = 7.18–495) or with CIN3 (OR = 40.0;95% CI = 8.07–198), there was no association between CIN3 and high levels of IL-10 when compared to women with no lesions (OR = 0.63;95% CI = 0.07–5.75). These observations indicate that increased levels of IL-10 occur in late events of the carcinogenic process or as a result of invasion; meanwhile IFN-γ seems to have a more important role by preventing the establishment of persistent infection [31].

Table 3
Bivariate analysis of the association of levels of polyamines with SCC and CIN 3 in women from Medellin—Colombia, 2007–2010. Bold numbers denote statistically significant values.

Factor	Controls (N = 79)	CIN3 (N = 41)	SCC (N = 84)	CIN3 vs controls	SCC vs controls	SCC vs CIN3
	n (%)	n (%)	n (%)	OR[a] (95% CI)	OR[a] (95% CI)	OR[a] (95% CI)
ASE pathway						
Arginase activity (nmol)						
Others	72 (91.1)	18 (43.9)	46 (54.8)	1	1	1
Upper tertile (>29.8)	7 (8.9)	23 (56.1)	38 (45.2)	**24.3(3.82–155.0)**	**9.8 (2.34–40.8)**	0.63 (0.27–1.45)
L-Arg(μM)						
Others	27(34.6)	38 (92.7)	70 (83.3)	1	1	1
Upper tertile (>126.3)	51(65.4)	3 (7.3)	14 (16.7)	**0.03 (0.004–0.19)**	**0.06 (0.02–0.24)**	2.99 (0.62–14.5)
L-Ornithine (μM)						
Others	45 (57.7)	39 (95.1)	51 (60.7)	1	1	1
Upper tertile (>104.1)	33 (42.3)	2 (4.9)	33 (39.3)	0.18 (0.03–1.16)	0.64 (0.22–1.86)	**10.3 (2.21–48.1)**
Putrescine (μM)						
Others	75 (96.2)	38 (92.7)	22 (26.2)	1	1	1
Upper tertile (>0.1)	3 (3.9)	3 (7.3)	62 (73.8)	1.79(0.10–33.2)	**149 (15.6–1420)**	**32.9 (8.81–123)**
Spermine (μM)						
Others	78(100.0)	2 (4.9)	55 (65.5)	1[b]	1[b]	1
Upper tertile (>624.2)	0.5(0.0)	39 (95.1)	29 (34.5)	**3042(133.95–69,082)**	**82.3(4.92–1376)**	**0.01 (0.001–0.11)**

Exposure variable: >upper tertile.
Bold numbers denote statistically significant values.
[a] Adjusted by age and HPV status.
[b] OR was not adjusted because limited sample size; zero was replaced by 0.5 in these cells to estimate the crude OR.

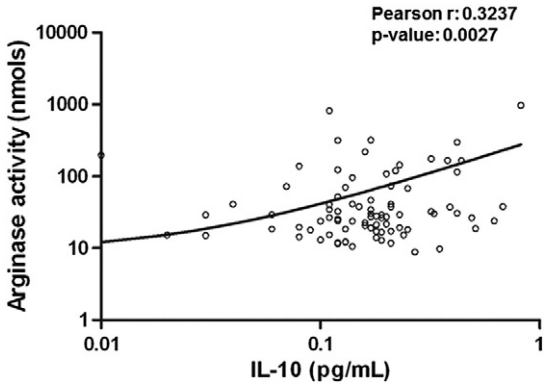

Fig. 2. Correlation between IL-10 levels and arginase activity among women with squamous cell carcinoma (SCC). Levels of IL-10 are shown in a logarithmic scale. Pearson and Spearman coefficient is shown.

In the last five years, ASE-induced L-Arg deprivation is emerging as a key mechanism for the down-regulation of immune responses not only in cancer but also in different infectious diseases [32,33]. It is also well documented that the expression of ASE and NOS2, key elements of the regulation of tumor promotion and cytolysis are induced by Th2- and Th1-type cytokines respectively [7,8]. Therefore we postulated that since there is a polarization towards Th2-type immune response associated with increased risk of cervical cancer, these patients may also exhibit increased levels of ASE activity with a consequent decrease of L-Arg and a pattern consistent with the interference of the corresponding pathway metabolites. Remarkably, we observed that high levels of ASE activity and low levels of L-Arg were associated with CIN3 and SCC. Our study presents evidence that these effects may seem to be regulated by Th2-type cytokines. We observed a statistically significant correlation between increased levels of IL-10 and high levels of ASE activity (Fig. 2, Pearson $r = 0.32$, $p = 0.0027$). The high levels of ASE activity with concomitant immunosuppression observed in cervical cancer may be supported by previous in vitro observations. Lepique et al. characterized tumor-associated macrophages that were obtained from a mouse model expressing E6 and E7 HPV-16 oncoproteins. In this study, these cells showed a high ASE activity, high IL-10 production and did not display NOS2 activity even after activation with IFN-γ [34]. It has been observed that tumor lines and myeloid suppressor cells that infiltrate the tumors [35,36] can produce ASE. There has been also an observation that HPV-specific CD4 + and CD8 + T cells within cervical cancer environment are functionally inactive [37]. Taken together, these and our observations, may suggest that the immunosuppressive microenvironment observed during human cervical carcinogenesis may at least be partially due to the ASE activity and concomitant degradation of L-Arg.

There is evidence that certain cofactors that alter the risk of cervical cancer may change the pattern of cytokine expression in the cervix of healthy women. Based on these observations, we conducted an interaction analysis. The interaction occurs between two risk factors when the effect of one factor (number of sexual partners, cigarette and contraceptive use) on the disease (cervical cancer) changes, when comparing the risk associated with different levels of exposure of a second risk factor (cytokine levels).When interaction is not present, the effect of the first risk factor on disease is homogeneous across levels of the second risk factor. In this analysis a synergism was observed in women with high levels of IL-10; as the risk of cancer is much higher in women with more than two sexual partners and high levels of IL-10 compared with women who also have high levels of IL-10 but only reported one lifetime sexual partner. The number of sexual partners may increase the risk of acquiring other sexually transmitted infections and modify non-specific cytokine levels in the mucosa of the cervix and in the serum, which in turn may interfere with the type and quality of the immune response against HPV.

A potential limitation of our study is that the measurement of cytokines and L-Arg metabolite levels in cervical cancer in the presence of HPV, which also can biologically modulate these immune responses, can be confounded by risk factors associated with HPV infection. To address this issue we adjusted for age and HPV all logistic regression and interaction models, still however we cannot exclude the possibility of residual confounding by other non-measure risk factors. These findings should be corroborated in properly designed case–control and cohort studies. Although the evaluation of ASE activity is an accurate and direct evidence of the activity on the enzyme in vivo, future studies should use specific antibody-based assays to distinguish if ASE 1 or ASE 2 is responsible for the degradation of L-Arg in women with cervical cancer. To the best of our knowledge, this is the first evidence suggesting the role of ASE and depletion of L-Arg associated with cervical cancer. Our results have serious implications in the understanding of the natural history of cervical cancer, and the potential application of the findings to foster the research in the development of therapeutic targets. Myeloid suppressor cells infiltrating the tumors [7,38] or in peripheral blood, seem to be the source of ASE, therefore the findings of our study provide support and suggest that it is worth studying a possible role of this type of cells as a mechanism for the inhibition of the capacity of T $CD8^+$

Table 4
ORs for interaction between cigarette smoking, contraceptive use and number of sexual partners with cytokine levels and cervical cancer in women of Medellin—Colombia 2007–2010. Bold numbers denote statistically significant values.

Cytokines	Cigarette smoking			Contraceptive use			Number of sexual partners			HPV infection		
	Never	Ever	p-Value**	Never	Ever	p-Value**	1	2 +	p-Value**	Negative	Positive	p-Value***
IFN-γ												
Others	1	1.03	0.653	1	0.16	0.058	1	8.51	0.703	1	228	0.516
Upper tertile (>1.37)	0.01	0.02		0.00	0.01		0.02	0.11		0.00000001	4.23	
IL-12p70												
Others	1	2.10	0.720	1	0.78	0.374	1	9.37	0.837	1	160	0.641
Upper tertile (>0.86)	0.01	0.04		0.004	0.02		0.02	0.11		0. 00000001	1.96	
IL-10												
Others	1	3.17	0.802	1	2.87	0.130	1	74.7	**0.024**	1	966,658,149	0.103
Upper tertile (>0.12)	67.9	152		223	75.6		1076	1247		278,603,339	15,328,625,887	
IL-13												
Others	1	2.78	0.306	1	0.73	0.127	1	7.03	0.568	1	113	0.279
Upper tertile (>0)	2.44	23.9		0.96	4.78		2.17	33.5		7.54	239	
IL-4												
Others	1	1.87	0.725	1	0.71	0.550	1	11.0	0.363	1	107	0.385
Upper tertile (>0.06)	0.03	0.03		0.01	0.02		0.07	0.17		0.000000009	2.77	

p value for interaction: **Chi-square test of deviance, adjusted by age and HPV. ***Chi-square test of deviance, adjusted by age.
Bold numbers denote statistically significant values.

lymphocytes to eliminate HPV-infected cells. It has also been demonstrated that targeted depletion of polyamines induces arrest in cell growth of Hela cells. Even further, vectors expressing the spermidine/spermine N1-acetyltransferase 1 (SAT1), the key regulatory enzyme in the catabolism of polyamines, seem to have utility for devising tumor-targeted gene therapy to overcome the inhibitory effect of ASE [39,40].

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Conflict of interest statement

The authors declare that they have no competing interests.

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