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# Interleukin-17 expression in the serum and exfoliated cervical cells of patients infected with high-risk oncogenic human papillomavirus

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ABSTRACT

Persistent infection by high-risk oncogenic human papillomavirus (HR-HPV) is the main cause of cervical cancer and its precursor lesions, and both the systemic and local immunological responses play an important role in eliminating or maintenance this infection. Th17 cells, as well as interleukin (IL)-17, are related to tumor growth and persistence of viral infection. Thus, this study aimed to quantify IL-17 in the serum and exfoliated cervical cells of HR-HPV-infected patients and healthy patients as well as identify $CD4^+IL17^+$ cells and IL-17 production in uterine cervix biopsies to better understand the behavior of this cytokine in HPV infections. IL-17 was quantified (pg/mL) in the serum and exfoliated cervical cells of 26 HR-HPV-infected patients, and in 18 healthy patients, using flow cytometry. Fifteen paraffin-embedded biopsy samples from the uterine cervix were subjected to immunohistochemistry to detect $CD4^+IL\text{-}17^+$ and $IL\text{-}17^+$ cells. There was a significant increase in the concentration of IL-17 in HR-HPV-positive patients' serum when compared to that in samples of exfoliated cervical cells ($p < 0.05$). Likewise, when compared with that in healthy patients, the IL-17 concentration was still higher in HR-HPV-positive patients sera ($p < 0.05$). We did not find differences in the amount of $CD4^+IL\text{-}17^+$ cells and other IL-17-secreting cells between different histopathological lesions. Our results suggest that HR-HPV infection predominantly stimulates systemic IL-17 production along with less localized expression.

## 1. Introduction

Interleukin 17 (IL-17) was discovered in 1993 and originally called cytotoxic T lymphocyte-associated-8 (CTLA-8) [1]. This cytokine is part of the IL-17 cytokine family that is composed of the following members: IL-17A (commonly called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F, where IL-17 and IL-17F are the best characterized [2,3].

IL-17, an important pro-inflammatory cytokine, is the signature cytokine of the Th17 response profile [4,5]. In addition to Th17 cells, a variety of cell types can produce IL-17, including γδ T cells [6,7], CD8+T cells [8], neutrophils [9,10], mast cells [10], NK [11], iNKT [12], monocytes [13], and ILC3 [14], indicating that IL-17 is an effector cytokine produced by innate and adaptive immune cells. The interaction of this cytokine with its receptors, widely expressed in endothelial and epithelial cells as well as fibroblasts, results in the release of cytokines (TNF-α and IL-1β) [15], chemokines (CXCL1, CXCL8, and CXCL10) [16], and metalloproteinases [17] that lead to inflammation and tissue damage [18].

In addition to being involved in the inflammatory process, it has been demonstrated that IL-17, secreted by CD4 T cells, can also promote neovascularization [19]. When acting upon fibroblasts and stromal cells, IL-17 induces several angiogenic mediators including vascular endothelial growth factor (VEGF), prostaglandins, and chemokines derived from keratinocytes that promote angiogenesis and tumor growth [19,20]. As demonstrated by Tartour *et al.* [21], IL-17 can also promote human cervical tumor growth through IL-6-dependent mechanisms. These results led to IL-17 and Th17 cells becoming the target of several studies involving cancer and tumors.

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The high density of Th17 cells infiltrated in tumors has been associated with increased angiogenicity in studies of patients with gastric [22], colorectal [23], hepatocellular [24], and pancreatic cancer [25]. Moreover, these cells were found in greater proportion in the peripheral blood of patients with cervical cancer [26], and their levels were positively correlated with the state of lymph node metastases and vain invasion [27]. Likewise, increased expression of IL-17 has been found in several tumor tissues including gastric, ovarian, and lung cancer [28–30]. Also, it has been demonstrated that increased expression of this cytokine is associated with growth of cervical cancer cells [21].

Cervical cancer is the fourth most common type of cancer among women worldwide. Current estimates indicate that 527,624 women each year are diagnosed with cervical cancer, and 265,672 die of this disease [31]. It is already well established that the main cause of cervical cancer and its precursor lesions (squamous intraepithelial lesions – SIL) is persistent infection by high-risk HPV (HR-HPV), mainly HPV 16 and 18 [32,33], and the individual's immunological response plays a crucial role in the viral elimination and control of lesion progression induced by this virus [34,35]. Bearing in mind that IL-17 and Th17 cells have been associated with the development of cervical cancer and/or its precursor lesions [21,26,36], the present study aimed to measure IL-17 levels in HR-HPV-positive patients peripheral blood and exfoliated cervical cells (ECCs), enabling a better understanding the role of this cytokine behavior at both sites.

## 2. Material and methods

### 2.1. Study population and specimen collection

Our study was initially composed of a cross-sectional study, aiming to form groups for the case-control study. Samples used in cross-sectional study were obtained from 410 female patients, aged over 18 years, who were attended at the Cancer Hospital of Barretos (CHB), Campo Grande – MS unit for cytological examination during the month of July 2016. All patients who agreed to participate in this study signed an Informed Consent Form. The present work was approved by the Research Ethics Committee of the Federal University of Mato Grosso do Sul and Pio XII Foundation under protocols n. 1468457 and 1635895, respectively.

The groups of the case-control study were composed of twenty-six HR-HPV infected patients with a mean age of 44.7 years and eighteen HPV DNA negative healthy patients (average 43.5 years). Both groups were negative for *Chlamydia trachomatis* and *Trichomonas vaginalis* DNA detection, negative in the anti-HIV and anti-*Treponema pallidum* antibodies screening, and negative for the circulating hepatitis B virus surface antigen (HBsAg), thus excluding systemic and local co-infections in the uterine cervix, which could influence the concentration of cytokines.

Samples of ECCs, peripheral blood, and uterine cervix biopsies were selected in a non-probabilistic way for convenience. The collection of ECCs was done before material collection for cytology in accordance with the literature [37–39]. ECCs were obtained through clinical collection by the hospital's own qualified professional by using a disposable speculum and soft endocervical brush with a protective tip. Two attached brushes were inserted in the cervical canal and rotated 5 times for material collection in the endocervix and ectocervix regions as well as the cervical mucus. Immediately after collection, the brushes were separated. One brush was intended for the identification and genotyping of HPV as well as the presence of co-infections, and it was stored in a sterile 15 mL tube. The other brush, intended for cytokine measurement, was placed in a 15 mL tube containing 1 mL of sterile PBS (pH 7.2). This tube was homogenized in a vortex, centrifuged, and the supernatant was aliquoted in a 0.5 mL tube for the determination of IL-17 levels. The material of both brushes was stored at −80 °C until processing.

Serum samples, intended for testing of cytokine levels and the identification of anti-HIV and anti-*Treponema pallidum* antibodies and HBsAg, were collected by venipuncture. Blood samples were processed to obtain serum, then aliquoted and stored at −80 °C until processing.

Uterine cervix biopsy samples used for immunohistochemistry (IHC) reactions were obtained from patients who presented with cytological alterations and were sent for histopathological examination. In addition to these samples, we included uterine cervix biopsy samples in the study with LSIL, HSIL, and CC histopathological classification stemming from prior studies [40] as immunological response assessment parameters in different degrees of lesions.

### 2.2. Detection of HPV infection

DNA was extracted from 410 clinical samples using the phenol-chloroform technique. After extraction, raw DNA was subjected to quantification in a BioDrop spectrophotometer.

The HPV DNA detection was performed by polymerase chain reaction (PCR) amplification with the use of PGMY09/11 primers targeting a 450-bp region of the HPV L1 genome as described in the literature [41]. As an endogenous control and to verify DNA integrity, primers PC04 and GH20 were used for the beta-globin gene, which amplify a 286-bp region of human DNA [42]. Positive samples in the reaction with primers for β-globin and PGMY09/11 were genotyped by type-specific PCR, using primers for high-risk HPV (HR-HPV) including HPV 16, 18, 31, 33, and 45 [43] as well as low-risk HPV (LR-HPV) including HPV 6 and 11 [44]. The PCR products were analyzed using 1.5% agarose gel electrophoresis with ethidium bromide staining to visualize DNA under ultraviolet light. Molecular weights were determined by comparison with a 100-bp DNA ladder. Restriction fragment length polymorphism (RFLP) was used for HPV DNA positive samples but with viral types undetermined by type-specific PCR (TS-PCR). The PGMY 09/ 11 PCR product of these samples was purified from agarose gel using a QIAEX II Gel Purification kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's protocol. The level of extracted material was measured using a BioDrop Spectrophotometer, and samples with sufficient material (≥4.0 ng/mL) were subjected to enzymatic digestion for 1 hr at 37 °C. Enzymes used for the reaction were *Dde*I, *Hae*III, *Rsa*I, and *Pst*I. The digestion pattern obtained was analyzed under UV light and interpreted using an algorithm described in the literature [45]. Samples that were not genotyped by TS-PCR and RFLP were sequenced.

In order to obtain the fragment to be sequenced, we performed a Nested-PCR using internal primers GP5+/GP6+ that amplify a 150-bp fragment of the L1 region [46]. The obtained product was purified with phenol-chloroform and dried at 60 °C, together with 6 μl of ultrapure water plus 1 μl of GP5+ primer. Tubes containing sample and primer were sent to ACTGene Molecular Analyses (Alvorada – RS/Brazil) for sequencing using ABI-Prism 3500 Genetic Analyzer equipment (Applied Biosystems, Foster City, California, USA).

### 2.3. Detection of Chlamydia trachomatis and Trichomonas vaginalis DNA as well as screening for anti-HIV, anti-Treponema pallidum and HBsAg

The detection of *Chlamydia trachomatis* and *Trichomonas vaginalis* DNA was carried out by conventional PCR using primers TVK3-5′ATT GTCGAACATTGGTCTTACCCTC3′ and TVK7-5′TCTGTGCCGTCTTCAA GTTAG3′ [47], and primers KL1-5′ TCCGGAGCGAGTTACGAAGA 3′ and KL2-5′ AATCAATGCCCGGGATTGGT 3′ [48], respectively. We analyzed PCR products using 1.5% agarose gel electrophoresis with ethidium bromide staining to visualize DNA under ultraviolet light. Molecular weights were determined by comparison with a 100-bp DNA ladder.

For the screening of infections caused by HIV, HBV, and *Treponema pallidum*, we used the following rapid tests: HIV Test Bioeasy (Standard Diagnostics, Inc., South Korea), Vikia® HBsAg (Biomerieux, France),

and TR DPP Sífilis DUO Bio Manguinhos (Oswaldo Cruz Foundation, Brazil), respectively. All three tests are qualitative tests based on immunochromatographic techniques and approved by the Brazilian National Health Surveillance Agency (ANVISA), which is responsible for the regulation, control, and supervision of products and services that involve risk to public health.

### 2.4. Cytology and histopathology

Cytological and histopathological examinations were carried out in the Clinical Pathology Laboratory of the CHB, Barretos unit (SP-Brazil). Cervical cytology was carried out in all patients participating in the study using the BD SurePath™ liquid-based Papanicolaou test (BD, Formerly TriPath Imaging Inc., Burlington, NC, USA). Patients who presented alterations suggestive of a lesion in the cytological exam were referred for additional evaluation by colposcopy examination; when necessary, a cervical biopsy was performed for histopathological examination.

### 2.5. Cytokine measurements

The IL-17A measurement was carried out in the serum and exfoliated cervical cells from 26 HR-HPV infected patients and 18 healthy patients. The concentration of IL-17A cytokine was quantified using a Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA). The preparation of standards, beads, samples, and protocols for flow cytometer setup and data acquisition were performed according to the manufacturer's instructions. Samples were acquired using a FACS Canto II Flow Cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with the FCAP Array software V. 3.0 (Becton Dickinson, Franklin Lakes, NJ, USA). Results were based on standard concentration curves and expressed as pg/mL.

### 2.6. Immunohistochemistry

For IHC reactions, all uterine cervix biopsy samples from patients positive for HR-HPV DNA collected during this period were included in the present study (n = 4). In addition to these samples, we included biopsies with histopathological classifications of HSIL (n = 5), CA (n = 4), and LSIL (n = 2) from a previous study, obtained from patients seen in the Centre for Cancer Prevention of Campo Grande (MS-Brazil) [40].

We used immunohistochemistry on paraffin-embedded sections to identify co-stained with CD4 (Abcam, Cambridge, UK) and IL-17 (Abcam, Cambridge, UK) markers. Antigen retrieval was performed in a pressure cooker using 10 mM of sodium citrate (pH 6.0). Detection of immunohistochemical signals was performed using a Polink DS-MR-Hu A1 Kit (GBILabs, Bothell, WA, USA) according to the manufacturer's instructions. Slides were counterstained with Harris hematoxylin. Positive (with primary antibody) and negative controls (without primary antibody) were included using sections of human tonsil.

For sections subjected to immunohistochemistry, images were acquired using a Moticam 2300 digital camera coupled to a Nikon Eclipse E200 microscope (Nikon, Japan) and Motic Images Plus 2.0 software. Images (1024 × 768 pixels) were acquired from 10 randomly selected fields of view for each slide, at 400× magnification, without any knowledge of the grade of injury. The size of features in images were determined with ImageJ software 1.47 (National Institutes of Health, USA) using the freehand line tool. Images that were 1024 × 768 pixels corresponded to 170.67 × 128.00 μm. Using the color deconvolution plugin of ImageJ, the separation of stains was achieved as described by Ruifrok and Johnston [49]. Using the cell counter plugin of ImageJ software, the number of immunostained cells was determined for each image, providing the average number of immunostained cells per $mm^2$ [50].

**Table 1**
Characteristics of the study participants attended in the Cancer Hospital of Barretos, Campo Grande, MS, Brazil 9 (n = 410).

| Characteristics | n (%) |
|---|---|
| *Age* | |
| < 40 years old | 122 (29.7%) |
| 40–50 years old | 143 (34.8%) |
| ≥51 years old | 145 (35.4%) |
| *HPV Status* | |
| Infected | 37 (9.0%) |
| Non-infected | 373 (90.9%) |
| *Cytology* | |
| ASC-US | 7 (1.7%) |
| ASC-H | 1 (0.24%) |
| NILM | 400 (97.6%) |
| LSIL | 1 (0.24%) |
| HSIL | 1 (0.24%) |

ASC-US: Atypical Squamous Cells of Undetermined Significance; ASC-H: Atypical Squamous Cells where high-grade lesions cannot be excluded; NILM: Negative for intraepithelial lesion and malignancy; LSIL: Low-grade Squamous Intra-epithelial Lesions; HSIL: High-grade Squamous Intra-epithelial Lesions.

### 2.7. Statistical analysis

Values indicating the concentration of IL-17 are expressed as mean ± SEM. Data were analyzed using non-parametric tests. To test for a significant difference between groups, we used the Mann–Whitney *U* test. The Kruskal–Wallis test was used to compare the histopathological findings and results of immunohistochemistry. Results were considered significant if $p < 0.05$. All statistical analyses were performed using GraphPad Prism 5.0 (Graph-Pad Software, San Diego, CA, USA).

## 3. Results

Characteristics of the study participants are described in Table 1. Of 410 analyzed samples, 9% (37/410) were positive for HPV DNA. Among these samples, 8.1% (3/37) were positive for *Chlamydia trachomatis* DNA, 5.4% (2/37) were positive for detection of anti-*Treponema pallidum* antibodies, and 5.4% (2/37) were positive for detection of HBsAg, hence being excluded from the study.

### 3.1. Predominance of HR-HPV in exfoliated cervical cells

As demonstrated by Fig. 1, HR-HPV was detected in 86.7% (26/30) of positive HPV-DNA samples and LR-HPV in 43.3% (13/30). Of these samples, 13.3% (4/30) were infected with only LR-HPV while 56.7% (17/30) were infected with only HR-HPV, and 82.35% (14/17) were simple infections caused by only one type of HR-HPV. Infection caused by more than one viral type was detected in 40.0% (12/30) of samples (Fig. 1). Among HR-HPV, we genotyped HPV 16, 18, 33, 45, and 31 by TS-PCR, HPV 59, 52, 66, 26, 30, 82, 85, and 53 by RFLP, and HPV 66 and 69 by sequencing. Among LR-HPV, HPV 6/11 were genotyped by TS-PCR, HPV 32 by RFLP, and HPV 81 and 89 by sequencing.

### 3.2. Comparison of IL-17 levels in serum and ECCs

The IL-17A (pg/mL) measurement was carried out in the sera and exfoliated cervical cells of 26 HR-HPV-infected patients (average age − 44.7 years), and in 18 healthy patients (average age − 43.6 years).

We observed that in HR-HPV-positive patients there was a significant increase in the concentration of IL-17 in the serum (25.84 ± 5.71 pg/mL) when compared with that in exfoliated cervical cell samples (1.54 ± 0.42 pg/mL) ($p < 0.05$). When compared to that

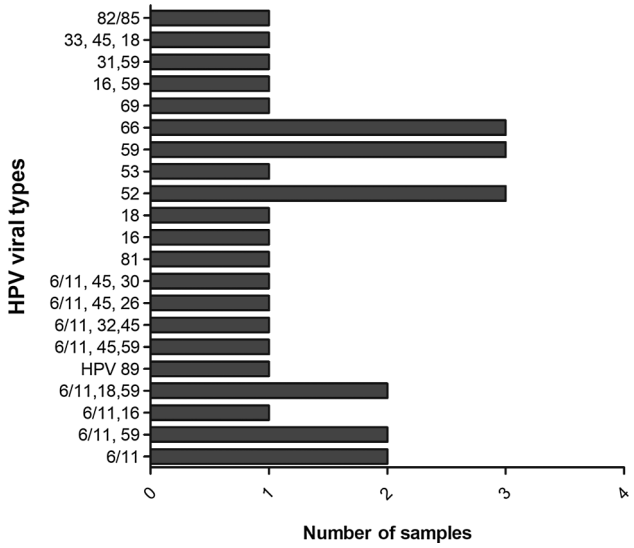


**Fig. 1.** Frequency of HPV genotypes.

in healthy patients, the concentration of IL-17 was also higher in the serum from patients positive for HR-HPV (5.73 ± 1.800 versus 25.84 ± 5.71 pg/mL, respectively) ($p < 0.05$). With regard to exfoliated cervical cells samples from HR-HPV-positive patients and healthy patients, there was almost no difference in the concentration of IL-17 (1.53 ± 0.42 versus 1.59 ± 0.35 pg/mL, respectively) ($p > 0.05$) (Fig. 2).

When comparing levels of IL-17 in the sera and ECCs of patients with a simple infection by only one type of HR-HPV, and multiple infection by more than one viral type, we observed that IL-17 levels in the serum (26.15 ± 9.27 versus 25.48 ± 6.47 pg/mL, respectively) and ECCs (1.85 ± 0.74 pg/mL versus 1.27 ± 0.46 pg/mL) were similar in samples with simple and multiple infection (Supplementary Fig. 1).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2019.04.008.

### 3.3. Cytology and histopathology

Among patients included in the cytokine analysis (n = 26), 21 were classified as negative for intraepithelial lesion or malignancy (NILM), 1 as low-grade squamous intraepithelial lesion (LSIL), and 4 as atypical squamous cells of undetermined significance (ASC-US). Five of those patients were sent for biopsy and histopathological analysis, four of them being classified as LSIL, and one as HSIL.

### 3.4. IL-17 and Th17 cells in uterine cervix lesions

Double-staining for CD4/IL-17 was carried out in 15 uterine cervix biopsy samples positive for HR-HPV, four of which came from patients attended in the Cancer Hospital of Barretos, Campo Grande unit, MS, and 11 coming from previous study [40]. Of the 15 samples, 5 were classified as LSIL, 6 were classified as HSIL, and 4 were classified as CC (Table 2).

We acquired 150 photos and analyzed them to determine the number of Th17 cells and other IL-17-secreting cells present in the stroma of uterine cervix biopsy samples (Supplementary Fig. 2). We observed that the mean of other IL-17-secreting cells ranged from 9898.95 to 12469.2 cells/mm$^2$, while the average of CD4 + IL-17 + cells ranged from 366.2 to 778.2 cells/mm$^2$, but did not vary significantly between different histopathological lesions (Supplementary Fig. 3). Although this finding was not significant, it is worth highlighting its importance, as the presence of IL-17, as well as the

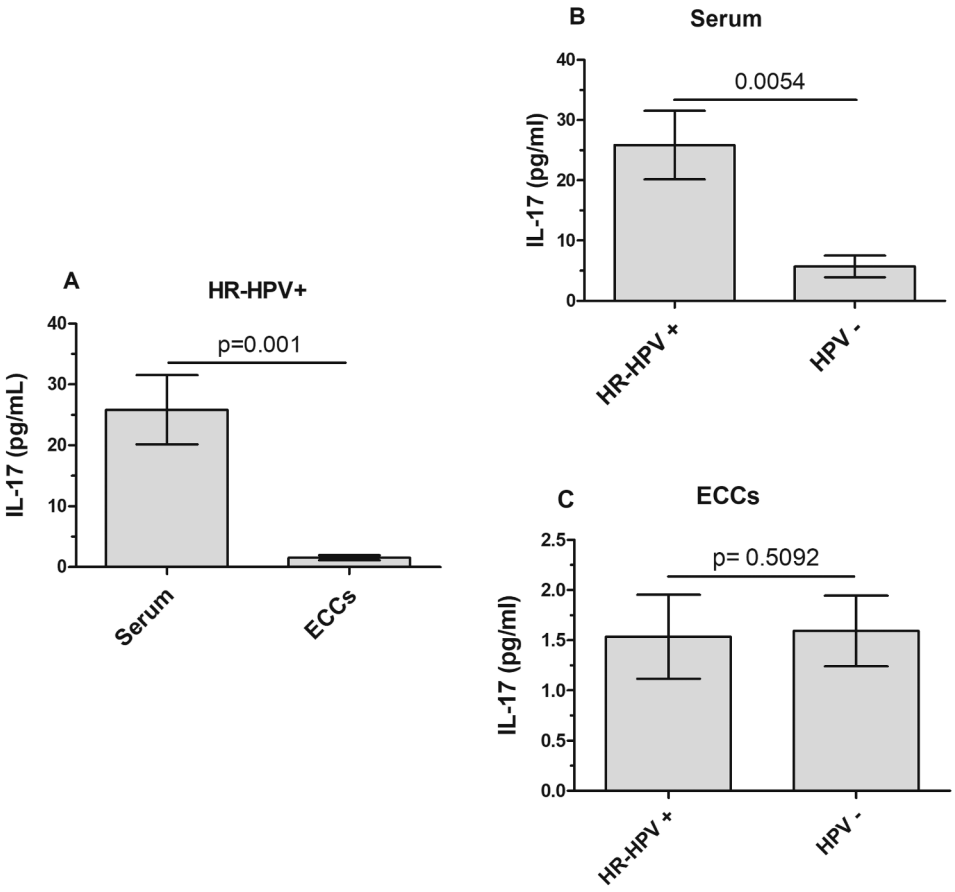


**Fig. 2.** Concentration of IL-17 in the serum and exfoliated cervical cells of HR-HPV-positive patients and healthy patients. (A) Concentration of IL-17 in the serum and exfoliated cervical cells of HR-HPV-positive patients. (B) Concentration of IL-17 in the serum of HR-HPV-positive patients and HPV-negative patients. (C) Concentration of IL-17 in exfoliated cervical cells of HR-HPV-positive patients and HPV-negative patients.

**Table 2**
Viral genotypes found in uterine cervix biopsy samples (n = 15) used in the immunohistochemistry.

| Genotypes | LSIL | | HSIL | | CC | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| HPV 16 | 1 | 20.0 | 1 | 16.7 | 1 | 25.0 |
| HPV 18 | 1 | 20.0 | 4 | 66.6 | 3 | 75.0 |
| HPV 59 | – | – | 1 | 16.7 | – | – |
| HPV 18, 59 and 6/11 | 1 | 20.0 | – | – | – | – |
| HPV 16, 59 and 6/11 | 1 | 20.0 | – | – | – | – |
| HPV 45 | 1 | 20.0 | – | – | – | – |
| Total | 5 | 100.0 | 6 | 100.0 | 4 | 100.0 |

LSIL: Low-grade Squamous Intra-epithelial Lesions; HSIL: High-grade Squamous Intra-epithelial Lesions; CC: Cervical Cancer.

expression of markers that characterize Th17 cells, were assessed at the site of viral infection.

## 4. Discussion

Infections by HPV in the female genital tract exhibit a transient pattern and most immunocompetent individuals (70–90%) are able to eliminate the virus in a period of 12–24 months after the initial diagnosis of infection [51]. However, in about 10% of cases, viral elimination does not occur, and the individual remains infected [33]. Several studies point out that the infection progression is associated with the persistence of HPV, presence of high-risk oncogenic types, high viral load, viral DNA integration in the cell, and immunological response failure [51–55].

The association of high-risk HPV with the development of cervical cancer and its precursor lesions has been demonstrated by several authors [32,33,56]. Among HPV types that contribute to cervical cancer development, HPV 16, 18, 58, 33, 45, 31, 52, 35, 59, 39, 51, and 56 are highlighted [56], with types 16 and 18 being responsible for about 70% of cases and the most prevalent in all geographical regions [56,57]. Although HPV 16 and 18 are the most relevant to cervical cancer, other genotypes have also been associated with this type of cancer and its precursor lesions [33,58,59]; furthermore, they may vary according to age and geographical region [56,57,60]. In our study, HR-HPV was present in 86.6% (26/30) of samples positive for HPV, HPV 59 and 45 being the most frequent (38.5% and 19.2%, respectively). Global epidemiologic studies indicate that 18 different high oncogenic risk HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) are associated with cervical cancer [57,61]. In our study, we also considered HPV 69, 30, and 85 classified as possibly carcinogenic [54], as high oncogenic risk.

Cellular immunity failure seems to facilitate the development of HPV-associated lesions, as demonstrated by authors who found that generalized deficiency of T cells was associated with higher incidence of anogenital neoplasia and an increased relative risk for cervical cancer by 5.4 times [62,63]. These findings reinforce evidence that immunosuppression exerts an important role in the persistence of HR-HPV infection and the epithelial hyperplasia associated with those viruses [64].

Th17 cells have been associated with the rise of viral persistence and inhibition of T cell cytotoxity in the chronic viral infection model. This finding is due, in part, to the action of IL-17, as it was demonstrated that this cytokine interferes in the $CD8^+$ T cell cytotoxic function by blocking the Fas-FasL pathway [65]. In cases of persistent HPV infection associated with high levels of IL-17, this cytokine may be contributing to the greater survival of keratinocytes infected from the Fas-FasL pathway blocking, providing viral persistence. Another important point to be highlighted is the immunosuppression mediated by IL-17 in HPV-associated epithelial hyperplasia, as demonstrated by Gosmann *et al.* [66]. They suggested that blocking IL-17 could be of therapeutic use in persistent infection by high oncogenic risk HPV, which would help prevent the progression of premalignant lesions in cancer [66].

In our study, IL-17 levels were significantly higher in the sera of HR-HPV-positive patients when compared with that in healthy patients ($p = 0.0054$), suggesting that viral infection somehow provides a favorable condition for secretion of this cytokine. However, further studies are necessary in order to follow the progression of infection and determine whether there will be viral elimination or the high levels of IL-17 will contribute to the persistence of infection.

When we compared IL-17 levels in ECCs of HR-HPV-positive patients with cytokine levels in healthy patients, we observed that the average concentration of IL-17 was similar in both sites. Conversely, when Marks *et al.* [38] evaluated the concentration of pro- and anti-inflammatory cytokines and chemokines in the cervical secretion of perimenopausal women infected by HR-HPV, they found increased levels of IL-17 when compared to that in HR-HPV-negative women, showing that there may also be an increase in the local production of this cytokine. In our study, there was no difference in the concentration of IL-17 in ECCs between different age groups, leaving other parameters that could be investigated in future studies such as viral load and hormonal changes.

Our main objective in the present study was to assess the concentration of IL-17 both in patients infected by HPV 16 and 18, and by other high-risk HPV, with the aim to observe the IL-17 behavior at systemic and local levels. We observed that levels of this cytokine were significantly higher in the sera of HR-HPV-positive patients when compared with that in the cervical microenvironment infected in these same patients, showing that there is a higher production of IL-17 at the systemic level. Stimulation of IL-17 production in the microenvironment may have been compromised by different immune response evasion mechanisms exerted by that virus including the negative regulation of TLR9 expression by viral proteins E6 and E7 [67] that impedes the innate immune response activation and subregulation of MHC I expression [68] by E5, which may also affect presentation via MHC II, essential for the activation of CD4+ T cells [69]. These mechanisms compromise the APC action in triggering of the cell-mediated immune response, allowing cells to remain HPV-infected.

A result discordant with that found in our study was reported by Xue *et al.* [36], who assessed the concentration of IL-17 in the serum and cervical tissue homogenate of HR-HPV-infected patients with normal cervical cytology, and observed that it was significantly higher for tissue homogenate when compared with that in serum. We have to consider that such results arise from the type of sample used for the performance of cytokine measurement. The sample material used in our study was obtained superficially with the collection of epithelial cells that flaked off from the endocervix and ectocervix during brushing and from the cervical mucus present in that site. Xue *et al.* [36] used cervical tissue homogenate that generally contains epithelial and stromal cells, thus widening the number of cells capable of secreting IL-17.

Vidal et al. [70], assessing cytokines and chemokines in the cervical tissue of patients with invasive cervical cancer infected by HPV 16 and 18 as well as other genotypes, observed low levels of IL-17 and high levels of IL-10, IL-15, and GMCSF in patients infected by HPV genotypes other than HPV 16 and 18, suggesting that different genotypes may stimulate different immune responses. Our results corroborate these data, considering that most viral genotypes detected were other than HPV 16 and 18. With regard to the cervix sample adequacy for cytokine levels, and considering that it could influence obtained concentrations, we report that a similar cervical sample collection methodology was previously carried out and consolidated by other studies [37–39].

Most studies attempting to clarify the relationship between HPV infection and the immunological response are carried out in patients with already established lesions, as in the study carried out by Xue et al. [36] who assessed the immune response in the cervical tissue homogenate and peripheral blood of patients with cervical intraepithelial

neoplasia (CIN) grade I, II, III, and cervical squamous cell carcinoma (SCC). They observed that the number of Th17 cells in blood and IL-17 levels in the cervical tissue homogenate supernatant gradually increased depending on the degree of cervical lesion. In our study, most patients did not present cytological changes; hence, to better understand IL-17 production in the local microenvironment, in addition to the four biopsies of patients involved in the present study, we used 11 more cases from a prior study [40] classified as LSIL, HSIL, and CC. Evaluating results for all samples, we demonstrated through immunohistochemistry that $CD4^+IL\text{-}17^+$ cells, as well as other IL-17-secreting cells ($CD4^-IL\text{-}17^+$), did not vary significantly between histopathological lesions.

Our results indicate that there is a strong stimulation of systemic IL-17 production in patients infected by high-risk HPV, suggesting that there is antigen presentation to cells, for instance naive Th17, present in subjacent lymph nodes. There is controversy associated with regard to the protumoral and antitumoral effects played by IL-17 and Th17 cells [71,72]. However, studies have related this cytokine to the development of cervical cancer and/or its precursor lesions [21,26,36] and to immunosuppression in HPV-associated epithelial hyperplasia [66]. Given that our study samples came from patients, typically without neoplastic lesions, the high levels of IL-17 found in serum may influence viral persistence through immunosuppressive mechanisms and contribute to neoplastic progression. However, additional studies are necessary in order to follow the evolution of infection in these patients.

## 5. Financial support

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## Author contributions

CMB, IAT and CTJP conceived the study. AFSP, LZAL and CMB collected the samples. ACB performed the colposcopy, biopsy and follow-up of patients, CMB, LZAL, ARS and AMTF performed the experiments. JCPR coordinated the analysis of samples in Barretos, SP, Brazil. CESF, IAT and CMB analyzed the data. CMB, CTJP and IAT drafted the manuscript. All authors have read and approved the final manuscript.

## Conflict of interest

The authors declared that they have no conflicts of interests.

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