International Journal of Women's Health

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

High-Grade Cervical Intraepithelial Neoplasia: Impact of Colposcopic Lesion Area on Systemic Immune Responses

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Background: The progression of high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer is accompanied by a reduction in the immune response. The objectives of the study were to determine whether colposcopic lesion area is associated with serum levels of cytokines IL (interleukin) −1, IL-6, IL-8, IL-10, IL-12 and TNF-α in precursor lesions of cervical cancer.

Methods: The study population comprised patients with high-grade squamous intraepithelial lesion who had undergone colposcopy, cervical biopsy, and measurements of serum cytokines by ELISA *(Enzyme-Linked Immunosorbent Assay)*. Genotyping for HPV (human papillomavirus) 16, 18, 45 and 52 was performed by PCR (*Polymerase Chain Reaction*). ROC (*Receiver Operating Characteristic*) curves were calculated to determine whether there existed a cut-off value for serum cytokines in patients with colposcopic lesion area smaller vs larger than 1 cm^2. For cytokines with significant results, these cut-off values were used to perform the multivariable analysis.

Results: There were 71 patients with CIN 2/3. ROC curves were calculated to verify a cut-off value for serum cytokine levels that could be used to distinguish between lesion areas <1 cm2 vs ≥1 cm2. Values with statistical significance were IL-1 >13.3 pg/mL and IL-12 ≤349.6 pg/mL. In the multivariable analysis, the independent variables associated with colposcopic lesion area greater than 1cm^2 were IL-1>13.3 pg/mL and IL-12 ≤349.6 pg/mL [OR (95% CI) = 10.10 (1.50–67.96); OR (95% CI)=10.70 (1.17–97.45), respectively].

Conclusion: Although CIN 2/3 is a local uterine cervix lesion, there is a systemic immunological response. Our results are unprecedented and could be the target of new important studies in public health and cervical cancer prevention.

Keywords: uterine cervical dysplasia, cytokines, uterine cervical neoplasms

Introduction

Cervical cancer has a high prevalence and is therefore considered a global public health problem.It is the fourth most common cancer among women worldwide.[1] The main risk factor for cervical cancer is human papillomavirus (HPV) infection. However, HPV infection alone is not sufficient for the development of cancer. The persistence of an oncogenic viral subtype may prove deadly in combination with genetic and environmental factors that promote cellular changes favorable to the emergence of pre-neoplastic lesions and their evolution.[2,3]

In this context, it is of fundamental importance to identify cervical intraepithelial neoplasia grades 2 and 3 (CIN), considered the true precursor lesion of cervical cancer.[4] The diagnosis and treatment of cervical lesions is based on the results of cervical pap smears, colposcopy, and targeted biopsy. Colposcopy provides a measurement of cervical lesion size, which may be used to predict the possibility of future procedures with negative biopsy results.[5]

Received: 25 October 2024
Accepted: 24 January 2025
Published: 10 February 2025

One of the primary factors determining the persistence or elimination of HPV infections and their evolution into pre-neoplastic lesions is the cellular immune response. The progression of cervical neoplastic lesions promotes changes in the pattern of cytokine secretion, with active participation of regulatory T lymphocytes, although the extent to which this occurs is not fully understood.[6]

Notably, expression of the E6 and E7 oncoproteins present in high-risk HPV is essential for the integration of HPV DNA into that of the host. These oncoproteins induce continuous cell proliferation, prevent apoptosis, and inactivate tumor cell suppression proteins (mainly p53 and retinoblastoma protein), resulting in infection progression. These oncoproteins also affect the innate immune response, interfering with the production of cytokines. Consequently, viral persistence and the accumulation of cellular changes allow for the development of high-grade lesions and tumor progression.[7]

The antitumor immune response requires an adequate balance in the production of cytokines; consequently, the progression or regression of lesions depends on the type and quantity of cytokines secreted in the body.[8] Studies suggest that the progression of high-grade intraepithelial neoplasia and cervical cancer is accompanied by a reduction in the immune response mediated by decreased Th1 activity and increased Th2 activity.[9,10] Increased TGF-β levels and decreased IL-2, IL-12 and TNF levels are associated with the development of intraepithelial neoplasias and cervical cancer.[11,12] Tumor progression is associated with increased levels of immunosuppressive cytokines such as IL-10, IL-4, and TGF-β.[13]

The most important therapeutic action to reduce cases of invasive neoplasia is treatment of high-grade cervical intraepithelial. This approach requires excision of the transformation zone containing the histological change, and can be performed using the Loop Electrosurgical Excision Procedure (LEEP), cold conization, and laser conization.[14]

The objectives of this study were to verify whether there is an association between colposcopic lesion area in patients with high-grade CIN and serum levels of cytokines IL-1, IL-6, IL-8, IL0, IL-12 and TNF-α.

Methods

This is a prospective cohort study from 2018 to 2024. The sample size calculation was performed using simple random sampling. A total of 128 patients were initially evaluated, with high-grade squamous intraepithelial lesion (HSIL) on pap smear at the Colposcopy and Gynecological Oncology Service. Subsequent cervical biopsy was performed to confirm high-grade CIN (CIN 2/3), and 71 patients were included in the study. Colposcopy and image archive, targeted cervical biopsy, genotyping for HPV (types 16, 18, 45 and 52), and serum collection for cytokine measurement were performed. Patients with immunosuppressive disease, pregnancy, and history of previous cervical procedure were excluded.

Patients with CIN 2/3 cervical biopsy were subsequently treated with LEEP, cold conization, or hysterectomy (only if there was no technical condition for conization, such as a flattened cervix). Decision-making about the type of procedure was based on individual patient criteria such as age and parity, as well as the anatomy of the cervix. The result of invasive carcinoma in the histopathology of the surgical specimen also was an exclusion criterion.

This study was approved by the Research Ethics Committee (CAAE 37116220.0.0000.5154, No. 4,597,976). All patients included in the study provided written informed consent.

Colposcopy and Measurement of Lesion Area

Colposcopy was performed according to guidelines provided by the International Federation for Cervical Pathology and Colposcopy-IFCPC 2011. Colposcopic images were archived and sequentially analyzed using the Image J program to determine lesion area in cm^2 (Figure 1).

Serum Collection for Cytokine Measurement

Serum collection was carried out on the day of the colposcopic examination. Venous blood samples were collected using a vacuum system and stored in tubes with separating gel (BD Vacutainer®). After 30 minutes of clotting, samples were centrifuged in a refrigerated centrifuge at 4°C for 10 minutes at 2000 rpm. Serum samples were stored in 250-µL aliquots at −70°C until use. Levels of IL-1, IL-6, IL-8, IL-10, IL-12 and TNF-α in plasma were determined by enzyme-linked immunosorbent assay (ELISA). Concentrations were calculated through comparison with their standard curves.

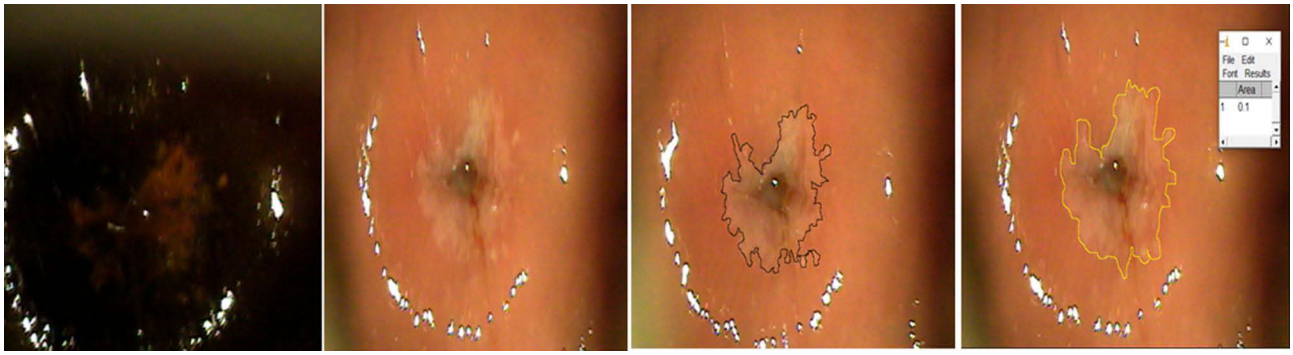

Figure 1 Assessment of the lesion area using Image J software.

Measurement of Serum Cytokine Levels

Serum levels of the cytokines IL-1, IL-6, IL-8, IL-10, IL-12, and TNF-α were determined by ELISA. Protein standards and samples for measurement were added to the wells of the ELISA plate in 100-µL aliquots. Standards and samples were coated with capture monoclonal antibodies for IL-1, IL-6, IL-8, IL-10, IL-12, and TNF-α. The ELISA plate was covered with adhesive sealant and incubated for 2 h at room temperature (RT) on a microplate shaker. Wells were aspirated and washed 5 times with 300 µL of buffer using automatic multichannel micropipettes. After the last wash, the plate was inverted onto absorbent paper to remove any residual buffer. Working solution (detection antibodies to IL-1, IL-6, IL-8, IL-10, IL-12, and TNF-α + streptavidin peroxidase conjugate) was added (100 µL per well), and samples were incubated for 1h at RT. Aspiration and washing were repeated as described above, then 100 µL of substrate solution (tetramethylbenzidine + hydrogen peroxide) were added to each well, and a third incubation was carried out for 30 minutes at RT in a plate microshaker completely protected from light. After incubation, 50 µL of stop solution (1M H3PO4) was added to each well. Finally, the optical density of each well was determined using a microplate reader set at 450 nm. The test has a detection limit of 1 pg/mL, inter-assay precision of 8–10%, and intra-assay precision of 4–6%. Concentrations were calculated by comparing them with their standard curves. Results are expressed as pg/mL.

Polymerase Chain Reaction (PCR)

Genotyping for HPV 16, 18, 45 and 52 was performed by PCR. Samples were stored in Trizol at −80°C and thawed when DNA extraction was accomplished. At this time, 200mL of chloroform was added for each 1.0mL of Trizol collected. DNA was added to an amplification solution according to the protocol suggested by the manufacturer (Invitrogen, Carlsbad, USA). Characteristics of the indicators synthesized to amplify specific DNA fragments (sequence, size of the amplified product and temperature annealing) were obtained according to Sarkar & Crissman (1990),[15] Tamim et al[16] and Dictor & Warenholt.[17]

Carrying out the PCR reaction, amplification products were subjected to electrophoresis in 14% polyacrylamide gels stained with silver. The Trackit 1 kB DNA ladder (Invitrogen, Carlsbad,USA) was used to estimate the size of the amplified product and the Beta-actina as a positive control of samples; 10.0mL of amplified sample and 3.0mL of buffer were homogenized and placed in each opening of 14% polyacrylamide gel. The gel was run at 90 volts for approximately one hour and then placed in fixative solution for 15 minutes. This solution was discarded and silver solution was added for 15 minutes, followed by washing in Milli-Q H2O and incubation in solution under development for approximately 15 minutes. The gel was returned to the fixative solution for 15 minutes, after which the resulting bands were observed.

Statistical Analysis

IBM SPSS Statistics and MedCalc software programs were used. Regarding the area of the lesion, we chose to use the cut-off value of 1 cm^2. ROC curves were calculated to determine whether there existed a cut-off value for serum cytokines in patients with colposcopic lesion area smaller vs larger than 1 cm^2. For cytokines with significant results, these cut-off values were used to perform the multivariable analysis, with the addition of age, smoking, estrogenic

contraceptive method, HPV type (16, 18, 45 and 52), and type of CIN as cofactors. Statistical significance was considered for p less than 0.05.

Results

The total number of patients referred to the Colposcopy Service with HSIL was 128. Of these, 71 patients met the inclusion criteria, with confirmation of CIN 2/3 on colposcopy-directed biopsy.

The median age was 33 years (18–68); median parity was 2 births (0–8); median age at sexual initiation was 16 years (10–23); median number of partners was 5 (1–21). Twenty-three patients (32.4%) were smokers. Thirty-three patients (46.5%) used hormonal methods for contraception; 15 (21.1%) had undergone a definitive surgical method; 4 (5.6%) used condoms; 1 (1.3%) used intrauterine device; and 18 (25.4%) did not use any method of contraception. There were 45 (63.4%) patients with positive HPV 16, 45 (63.4%) patients with positive HPV 18, 52 (73.2%) patients with positive HPV 45, and 45 (63.4%) patients with positive HPV 52 (Table 1).

Average lesion area was 0.87 mm^2. Therefore, we used the value of 1cm^2 as a cut-off value, because it is a value close to the average.

LEEP was the treatment in 41 cases (57.74%); only 18 (25.35%) and 6 (8.45%) patients were treated with conization and hysterectomy, respectively.

ROC curves were calculated to verify a cut-off value for serum cytokine levels that could be used to distinguish between lesion areas <1cm^2 vs ≥1cm^2. Cut-off values were found for the following serum cytokines: IL-1, >13.3 pg/mL (AUC=0.67 and p=0.024); IL-12, ≤349.6 pg/mL (AUC=0.663 and p=0.029). For the other cytokines, there were no statistical significances in the ROC curves (Figure 2).

In the multivariable analysis evaluating age, estrogen use, smoking, CIN grade, HPV type (16, 18, 45 and 52), IL-1 and IL-12 serum levels, the independent variables associated with colposcopic lesion area greater than 1cm^2 were IL-1>13.3 pg/mL and IL-12 ≤349.6 pg/mL [OR (95% CI) = 10.10 (1.50–67.96); OR (95% CI)=10.70 (1.17–97.45), respectively] (Table 2).

Table 1 Epidemiological and Clinical Data of Patients With HSIL

	Median, Minimum and Maximum Values and/or %
Age (years)	33 (18–68)
Parity	2 (0–8)
Age at sexarche	16 (10–23)
Number of partners	5 (1–21)
Smoking	23 (32.4%)
Contraception	
No method	18 (25.4%)
Hormonal method	33 (46.5%)
Tubal ligation	15 (21.1%)
Condom	4 (5.6%)
Intrauterine device	1 (1.3%)
Positive HPV 16	45 (63.4%)
Positive HPV 18	45 (63.4%)
Positive HPV 45	52 (73.2%)
Positive HPV 52	45 (63.4%)

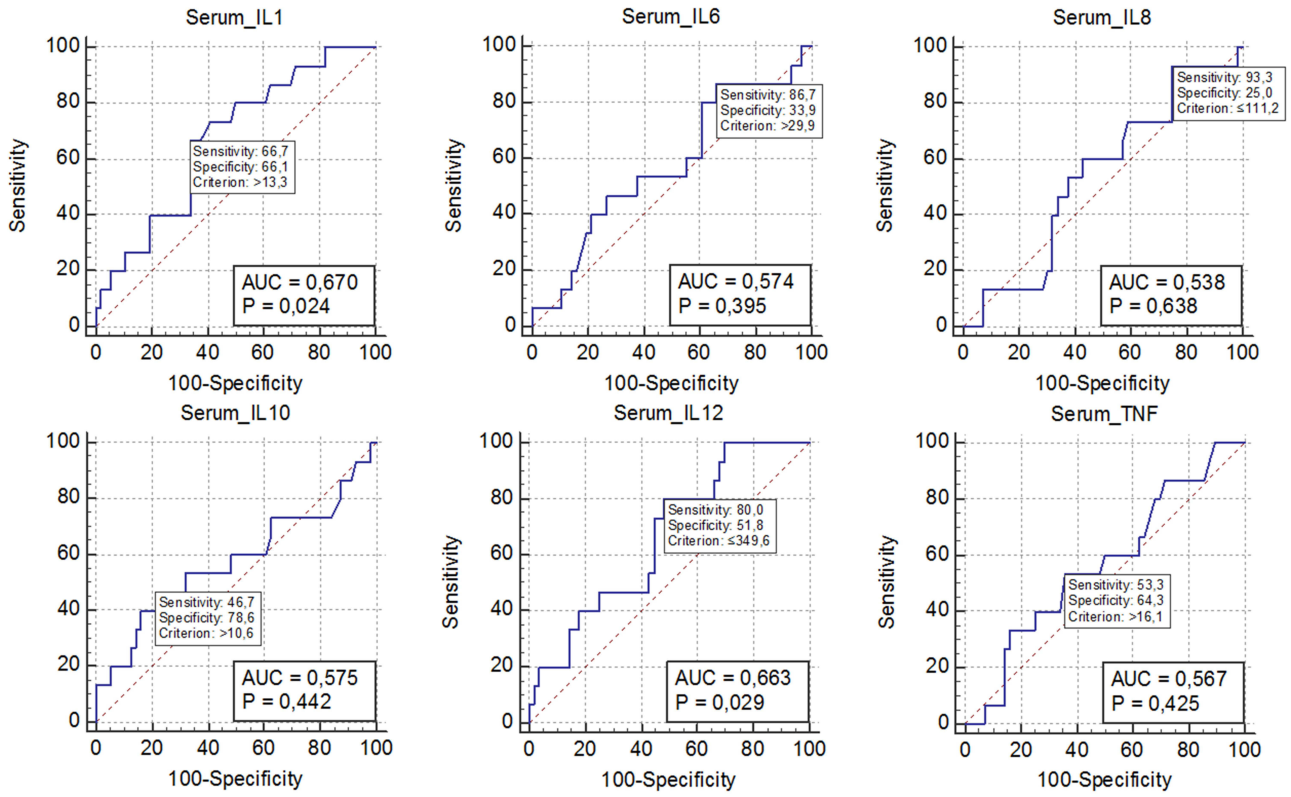

Figure 2 ROC curves showing serum levels of cytokines IL-1, IL-6, IL-8, IL-10, IL-12 and TNF-α in relation to colposcopic lesion area (greater or less than 1cm^2).

Discussion

The crucial role played by the immune response in eradicating HPV infections is evident. HPV modulates the production and secretion of cytokines by infected cells as a strategy to evade the immune system. Furthermore, the immune response can contribute to the establishment of a state of chronic inflammation, promoting the advancement of lesions associated with the virus and, consequently, the development of cancer.[18]

IL-1 is an inflammatory cytokine that plays a key role in carcinogenesis and tumor progression. It facilitates tumor initiation and progression, leading to a state of chronic inflammation without resolution, tumor angiogenesis, and induction of myeloid suppressor cells (MDSC), as well as the recruitment, invasion and metastasis of tumor-associated macrophages.[19] In our study, we observed that higher levels of IL-1 were present in patients with larger

Table 2 Univariate and Multivariate Analysis of the Variables Age, Parity, Estrogen Use, Smoking, CIN Grade, IL-1 and IL-12 Serum Levels Considering a Cut-off Value for the Colposcopic Lesion Area of 1cm^2

Variable	Univariate Analysis OR (95% CI)	Multivariate Analysis OR (95% CI)
Age (> 30y vs ≤ 30y)	3.90 (0.80–19.01)	6.93 (0.85–56.41)
Estrogen use (yes vs no)	1.46 (0.36–5.91)	5.38 (0.60–48.41)
Smoking (yes vs no)	0.48 (0.12–1.92)	0.12 (0.01–1.21)
CIN grade (CIN 3 vs CIN 2)	1.77 (0.35–8.96)	0.77 (0.07–8.11)
Serum IL-1 (> 13.3 pg/mL vs ≤ 13.3 pg/mL)	3.89 (1.16–13.03)	10.10 (1.50–67.96)
Serum IL-12 (≤ 349.6 pg/mL vs > 349.6 pg/mL)	4.30 (1.09–16.90)	10.70 (1.17–97.45)
Positive HPV 16	0.41 (0.13–1.32)	0.27 (0.05–1.47)
Positive HPV 18	0.83 (0.26–2.68)	1.73 (0.22–13.22)
Positive HPV 45	2.83 (0.58–13.95)	9.12 (0.69–121.06)
Positive HPV 52	0.71 (0.22–2.31)	0.21 (0.03–1.56)

https://doi.org/10.2147/IJWH.S503028

colposcopic cervical lesions. This finding may explain the pro-inflammatory role of IL-1 in the progression of CIN 2/3 lesions, especially in patients with lesions >1 cm2.

Inflammation and cancer are linked by an intrinsic pathway, whereby the genetic events that cause cancer orchestrate the construction of an inflammatory microenvironment, and an extrinsic pathway, whereby unresolved inflammation also leads to carcinogenesis.[20] As the inflammatory process is potentially pro-tumor, it predisposes cells to transformation through the induction of genomic instability and epigenetic changes, promotion of angiogenesis and cell proliferation, favoring tumor initiation and progression, in addition to the development of metastases.[21]

The contribution of IL-1 family members to the recruitment of immune system elements in response to damage or infection is linked to the homology between their receptors and Toll-like receptors (TLR). This similarity renders IL-1 family members significant elements of the innate immune response.[22,23]

It has been shown that IL-1 is upregulated in several types of tumors, such as breast, colon, head and neck, lung, pancreas, and melanomas.[24] IL-1 can be generated directly by cancer cells or can influence cells within the tumor microenvironment, stimulating them to produce it.[25] Patients with elevated IL-1 levels generally exhibit poor prognoses.[24]

In the literature, no articles were found that address the role of IL-1 in high-grade CIN. In our study, IL-1 > 13.3 pg/mL was an independent variable associated with colposcopic lesion area greater than 1 cm2. We believe that this finding is related to IL-1's pro-inflammatory and carcinogenic role.

Elevated concentrations of IL-1 in the tumor microenvironment have been documented in several studies, both in cancer patients and in experimental models, and are associated with a more aggressive tumor phenotype.[26] In certain tumor types, pro-inflammatory cytokines such as IL-1 can be upregulated by oncogenes, creating an environment conducive to tumor cell invasiveness. In contrast, in other tumor types, IL-1 is stimulated only during the late stage of cancer progression and metastasis formation.[27] The mechanisms underlying IL-1–mediated stimulation of metastases are complex and include the promotion of angiogenesis and the induction of endothelial cell adhesion molecules recognized by tumor cells.[26] IL-1 exerts complex effects on the activation of endothelial cells, directing them to a pro-thrombotic /pro-inflammatory response. This translates into the induction of procoagulant activity, as well as the expression of adhesion molecules and inflammatory cytokines.[19]

IL-12 is an antitumor cytokine that acts in antitumor therapies and in several immunotherapies.[28,29] In our study, IL-12 ≤ 349.6 pg/mL was an independent variable associated with colposcopic lesion area greater than 1 cm2. IL-12 acts as the main mediator between the innate and adaptive immune systems, controlling the proper development of naïve CD4 T cells into several T helper (Th) subunits.[30] In CD4+ T cells, IL-12 activates the transcription factor signal transducer and activator of transcription 4 (STAT4), which transcribes T-bet and differentiates Th1 cells. T-bet regulates the final expression of specific cytokines, Th1 chemokines, and specific receptors.[31] IL-12 also activates natural killer (NK) cells, which increase the expression of CD69 and CD25 in the tumor environment. Cytotoxic NK cells and CD8+ T cells secrete IFN-γ, perforin, and granzyme, which control the growth of a tumor and cause apoptosis of cancer cells.[32] IL-12 emits inflammatory danger signals to activate dendritic cells (DC) by tumor antigens, thereby reducing the induction of tumor tolerance in immune cells.[33]

Few studies in the literature address the role of IL-12 in HSIL. It is possible that its higher concentration related to minor colposcopic lesions can be related to its tumor control activity and apoptosis of cancer cells. A study demonstrated that, in addition to the multifunctional action that links adaptive and innate immunity, IL-12 is fundamental to the development of cellular immunity, with decreased IL-12 levels in the tumor microenvironment associated with lesion progression. Current research efforts have invested substantially in immunotherapeutic treatments against various types of cancers, include cervical cancer, because IL-12 has the potential to shift a Th1-dominant profile to a Th2-dominant profile, which is associated with immunomodulatory and antiangiogenic activity.[8]

This study has some limitations, including the small simple size and potential confounding effect of smoking. Small sample size can lead to very wide confidence intervals. Smoking is one of the most important cofactors in the progression of HPV-induced lesions and a potentially modifying agent, despite controversy in the literature, of the pattern of cytokine secretion. However, even adding smoking as a covariate in the multivariable analysis, the cytokines IL-1 and IL-12 remained independent variables associated with colposcopic lesion area greater than $1cm^2$. To date, the literature has not

established a relationship between colposcopic lesion area and magnitude of the systemic immune response in patients with CIN 2/3. The current study presents novel and promising results highlighting the need for adequate monitoring in cases with increased risk for disease progression. Furthermore, it suggests that the immune system is capable of establishing a systemic immune response in premalignant lesions of cervical cancer. Colposcopy is already a widely used method and measuring the area of the lesion can be carried out easily, without increasing examination time and causing patient discomfort. Systemic cytokine measurement is also an easy and non-invasive test, and can only be obtained by collecting peripheral blood. Therefore, these are methods that can be used even in the public health system.

Our results are unprecedented and could be the target of new important studies in public health and cervical cancer prevention. In the future, a better understanding of the systemic immune response triggered by cervical pre-neoplastic lesions may improve how to monitor lesion progression in cervical pre-cancerous lesions, and therefore help prevent cervical cancer.

Conclusion

Although CIN 2/3 is a local uterine cervix lesion, there is a systemic immunological response. Complementary studies, especially related to the profiles of IL-12 and IL-1 expression in patients with CIN, should be considered promising and of great importance in clinical practice.

Abbreviations

AUC, area under the curve; CD, cluster of differentiation; CIN,cervical intraepithelial neoplasia; DC, dendritic cells; ELISA, Enzyme-Linked Immunosorbent Assay; HPV, human papillomavirus; IFN, Interferon; IL, Interleukin; LEEP, Loop Electrosurgical Excision Procedure; NK, Natural Killer; OR, odds ratio; PCR, Polymerase chain reaction; Th, T helper; TGF, Transforming growth factor; TNF-α, Tumor Necrosis Factor.

Data Sharing Statement

Data available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the UFTM Research Ethics Committee (CAAE 37116220.0.0000.5154, number 4,597,976) and the patients signed the Consent Form. This study complies with the Declaration of Helsinki.

Acknowledgments

The authors wish to acknowledge the funding received from CNPq (National Council for Scientific and Technological Development - Brazil, process No.: 01115/2018-4 and research grant for Rosekeila Simões Nomelini process No.: 303086/2022-0), FAPEMIG (Minas Gerais Research Funding Foundation, process No.: APQ-00888-21 and RED-00011-14), FUNEPU (Uberaba's Teaching and Research Foundation, process No.: 02 2011), and CAPES (Brazilian Federal Agency for the Support and Evaluation of Graduate Education, process No.: 32012012001P5 and Proex).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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