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Original Research

Pegylated-interferon-alpha treatment modifying T cell cytokine profile in tumor microenvironment of patients with cervical intraepithelial neoplasia

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Objective: The goal of the investigation was to compare the profiles the cytokines IL-2, IL-12, IFN-γ, TNF-α , TGF-β, IL-4, IL-6 in the endocervical and serum secretions of patients with cervical intraepithelial neoplasia (CIN) who had good or poor response to immunotherapy with pegylated (PEG)-Interferon-alpha. *Methods*: The study was performed with 16 patients who had been diagnosed with CIN II and III. Each patient was submitted to six injections of PEG-Interferon-α -2b subcutaneously. After each injection, endocervical secretion and serum samples were collected for cytokine levels by ELISA. *Results*: Of the 16 women, 43.75% (n = 7) showed good clinical response and lesion regression after treatment as seen by histopathological analysis. When we analyzed the pre-therapy, locally we observed a higher concentration of IL-4 in patients who did not respond to treatment (P = 0.0229). After treatment, there was a significant reduction in IL-4 in unresponsive patients (P = 0.0304). Patients responsive to IFN-α had a reduction in the concentrations the TNF-α after treatment (P = 0.0313). *Conclusion*: Immunotherapy with PEG-Interferon-α can reduce the damage caused by HPV by decreasing local inflammation by reducing the cytokines IL-4 and TNF-alpha during treatment in endocervical secretion. It seems that the regression is related to the immunological profile, mainly local, before treatment, as in the case of IL-4 and TNF-alpha, promoting a Th2 profile in those patients with therapeutic failure.

Keywords

Cytokines; Immunotherapy; Cervical intraepithelial neoplasia

1. Introduction

Cervical cancer is among the leading causes of mortality worldwide and has human papillomavirus (HPV) as the underlying cause of its precursor lesions [1–3]. It remains a public health problem and is in fourth place among cancers in women with an incidence and mortality of 569,847 cases and 311,365 deaths reported worldwide [4].

The immune response to HPV is generally long-term [5], and has an important role in the course of infection both in its elimination and in its viral persistence leading to the development of cancer of the uterine cervix [6–8]. A recent study by Wang and his collaborators, demonstrated the importance of an early diagnosis of pre-malignant cervical lesions as screening tests for the prevention of cervical cancer [9].

The persistence of cellular lesions caused by human papillomavirus (HPV) depends on the ability of viral oncoproteins encoded by the viral E6 and E7 reading frames to manipulate the signaling pathways responsible for controlling cell proliferation and death, and mechanisms of innate immunity. Research shows that E6 / E7 inhibition can reactivate the host's innate immune response [10].

In addition, keratinocytes infected with high-risk HPV E6 / E7 oncoproteins have decreased STAT1α / β expression. STAT1 is activated in response to different cytokines, mainly interferons (IFNs), which may cause a reduction in the activation of immune cells, a tumor escape mechanism [11].

CD4+ T cells, called helper T lymphocytes, are subdivided into specific patterns, depending on the type of antigen and the cytokines produced, having an important role in specific immune responses. The response patterns related to these cells are: Th1 (main cytokines IL-12 and IFN-γ), Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) and Th17 (IL-1β, IL-6, IL-17, IL-22 and TNF-α), regulatory T cells (natural T reg, induced T reg, Tr1 cells and Tr3 cells) [12]. A cytokine secretion could also characterize the immune responses produced by diseases, including those associated with HPV [13, 14].

Because they have the ability to modulate response patterns against different aggressor agents in countless situations, cytokines can be used to treat various diseases [15]. The first cytokine to be used was interferon alpha (IFN-α) in immunotherapy against hepatitis C [16, 17]. Interferons are essential glycoproteins in defense against viruses, have an-

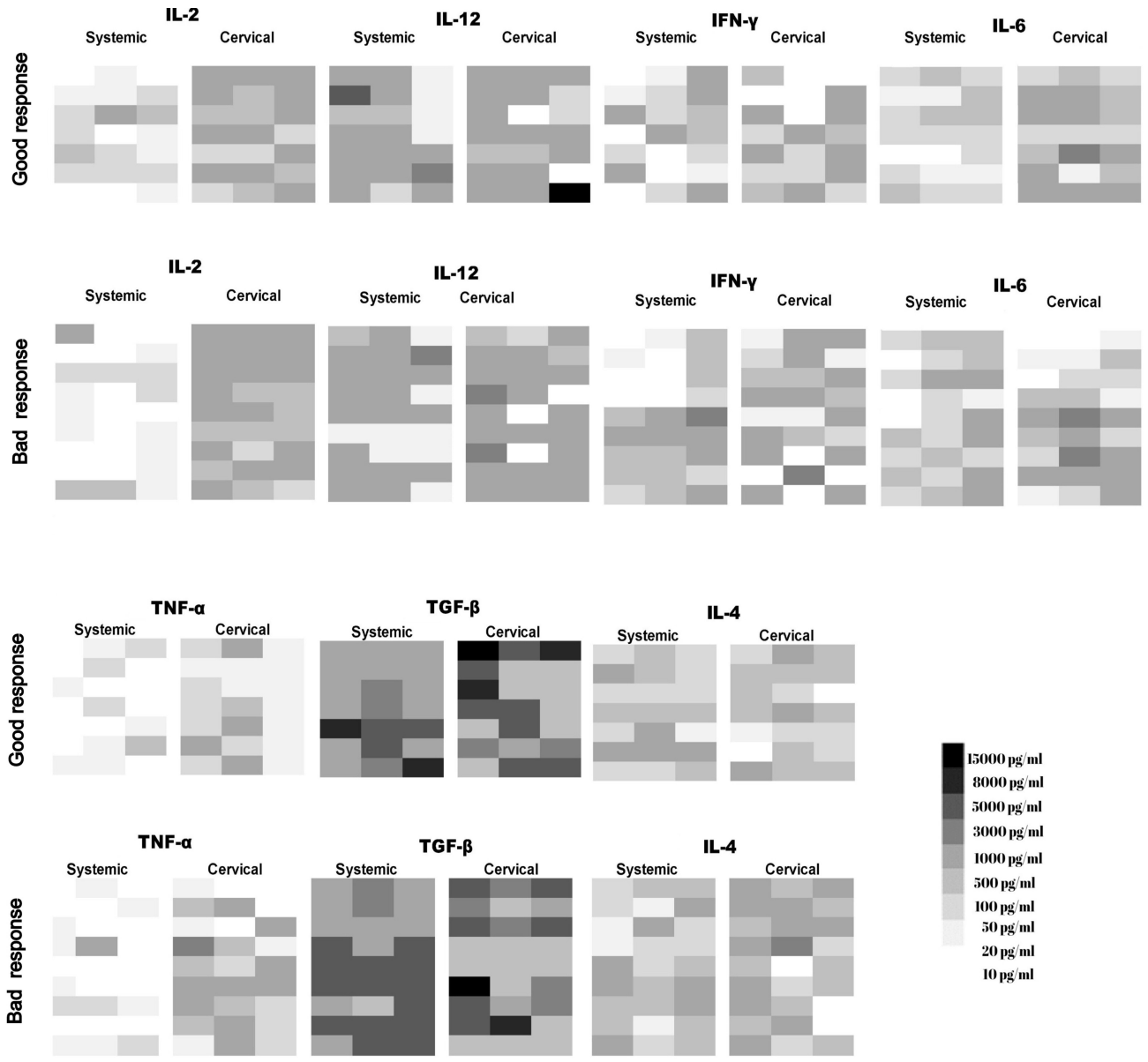

Fig. 1. Heat map illustrating the concentration of cytokines (IL-2, IL-12, IFN-γ, IL-6, TNF-α TGF-β and IL-4) in peripheral serum (systemic) and endocervical secretion (cervical) of patients with CIN II and III treated with IFN-alpha. Good response-Patients with regression of the lesion. Bad response-Patients without lesion regression. Dosage performed by ELISA technique.

tiproliferative and immunoregulatory activity, bind to specific receptors and interact directly with the cells to protect them against infection, as well as collaborate with the adaptive immune response [18, 19].

Knowing this important relationship between cytokines and immune response, several studies have used IFN-α as a therapy to fight viral infections such as HPV, as an alternative to several approaches that have been used as conventional in the treatment of intraepithelial neoplasia: conization, cryotherapy, laser vaporization and other surgical procedure [20–22]. A conservative treatment such as interferon therapy can bring great benefits to patients because it is an alternative for the treatment of the lesions caused by HPV by rescuing the immune system and thus, reduce the use of invasive procedures and consequent obstetric issues.

Studies have demonstrated a shift in the cytokine profile pattern towards the Th2 profile as the HPV-induced lesion worsens, and that the Th1 response is the most important in the control of HPV infection [23, 24].

This immunotherapy stimulates cell-mediated immune response that are essential to the elimination of HPV [25–29].

However, our group demonstrated in previous studies [26, 27, 30] that some patients with cervical intraepithelial neoplasia (CIN) respond to immunotherapy while others did not. Thus, it would be imperative to understand the local and systemic mechanisms of the performance of these cytokines in the regression of these neoplasms. To achieve that, the objective of this study was to compare the cytokines IL-2, IL-

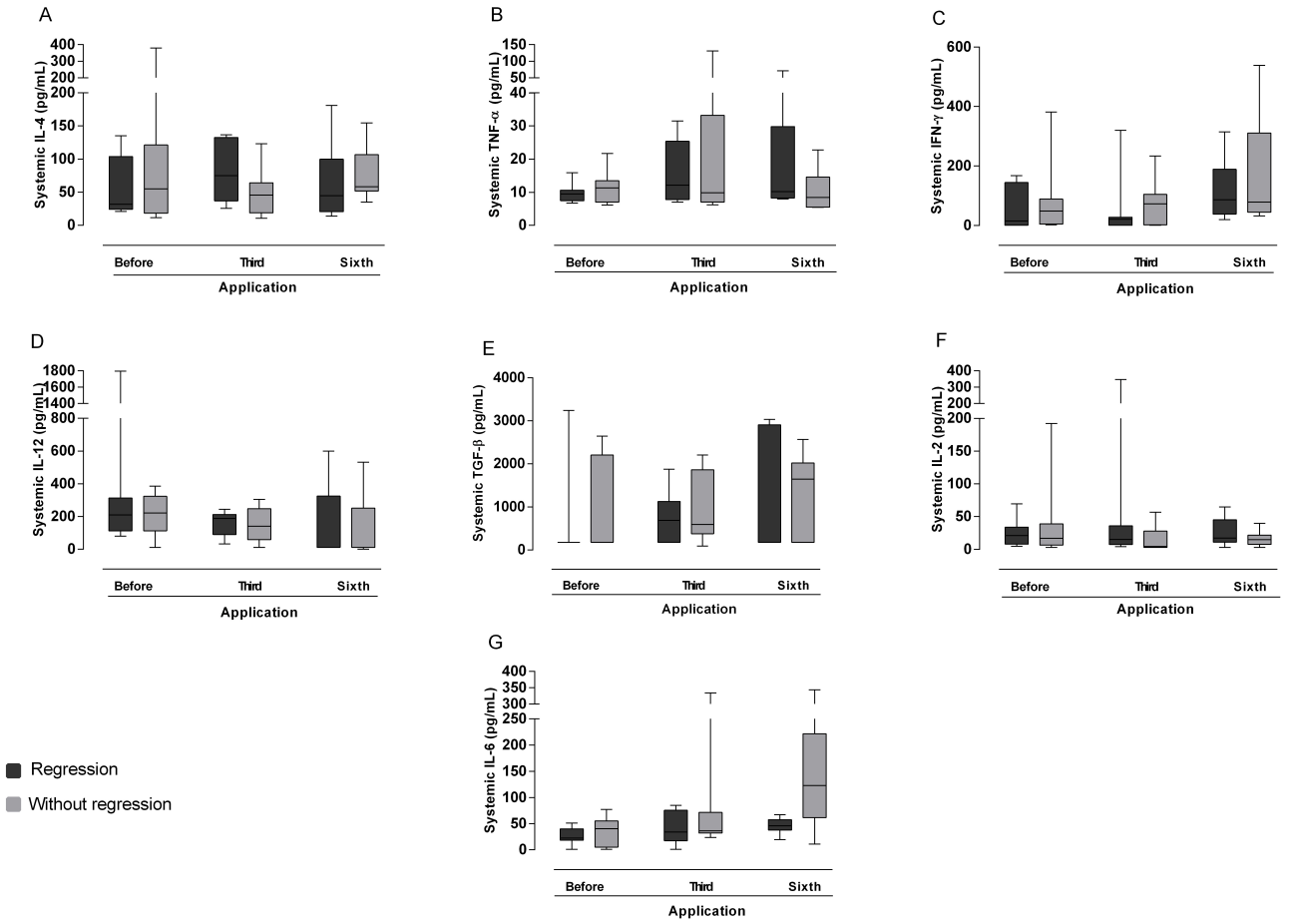

Fig. 2. Concentration of cytokines (IL-2, IL-12, IFN-γ, IL-6, TNF-α TGF-β and IL-4) in peripheral serum (systemic) of patients with CIN II and III during treatment with IFN-alpha. Dosage performed by ELISA technique and statistical analysis by the Mann Whitney test or Friedman test.

12, IFN-γ, TNF-α, TGF-β, IL-4 and IL-6 in the endocervical secretions and serum of patients who had good or poor response to pegylated (PEG)-Interferon-α.

2. Materials and methods

2.1 Patients

A prospective study was conducted at the Maria da Gloria Clinic, in the Gynecology and Obstetrics Discipline of the Federal University of the Triângulo Mineiro. From 2013 through 2015, 16 patients were included in the study ranging from 18 to 82 years of age, all diagnosed with CIN II-III, and who had not received any prior treatment. Our criteria for inclusion in the study was: the absence of bleeding during the examination; no sexual activity for two days preceding sample collection; no use of oral antibiotics, vaginal fungicides or creams over the previous 30 days; no previous history of treatment for HPV; and no colposcopic change $<$ 1 cm. The exclusion criteria included being smoker, having immunosuppressive diseases, serious heart disease, abnormal in liver or kidney function, pregnancy, a reported intolerance to IFN, or an absence of a visible lesion at colposcopy.

The clinical evaluation of the patients consisted of colposcopy and histological analysis. If colposcopy revealed regression and/or disappearance of the lesion, this was confirmed by histological analysis performed at the end of treatment. In the case of regression to CIN I or no CIN, these patients were included in the responsive group (good response). Patients were followed up with cytology and colposcopy every six months. If regression of the lesion was not observed on colposcopic examination, confirmation of the persistence of CIN II or III in biopsies was performed. These patients were included in the non-responsive group or treatment failure (poor response). All patients with CIN II and III were immediately submitted to cold knife conization. All patients were assessed for liver and kidney function.

The study was approved by the Research Ethics Committee of the institution (CEP / UFTM nº 759 and 1525) and the informed consent was obtained of all participants.

2.2 Collection of endocervical secretion

Endocervical secretion was collected weekly prior to IFN-peg applications by a licensed physician. For this procedure the exposure and inspection of the uterine cervix was performed with the introduction of a vaginal speculum and a brush to collect the material of the endocervix, and then rotating movements were performed. The secretory brush was

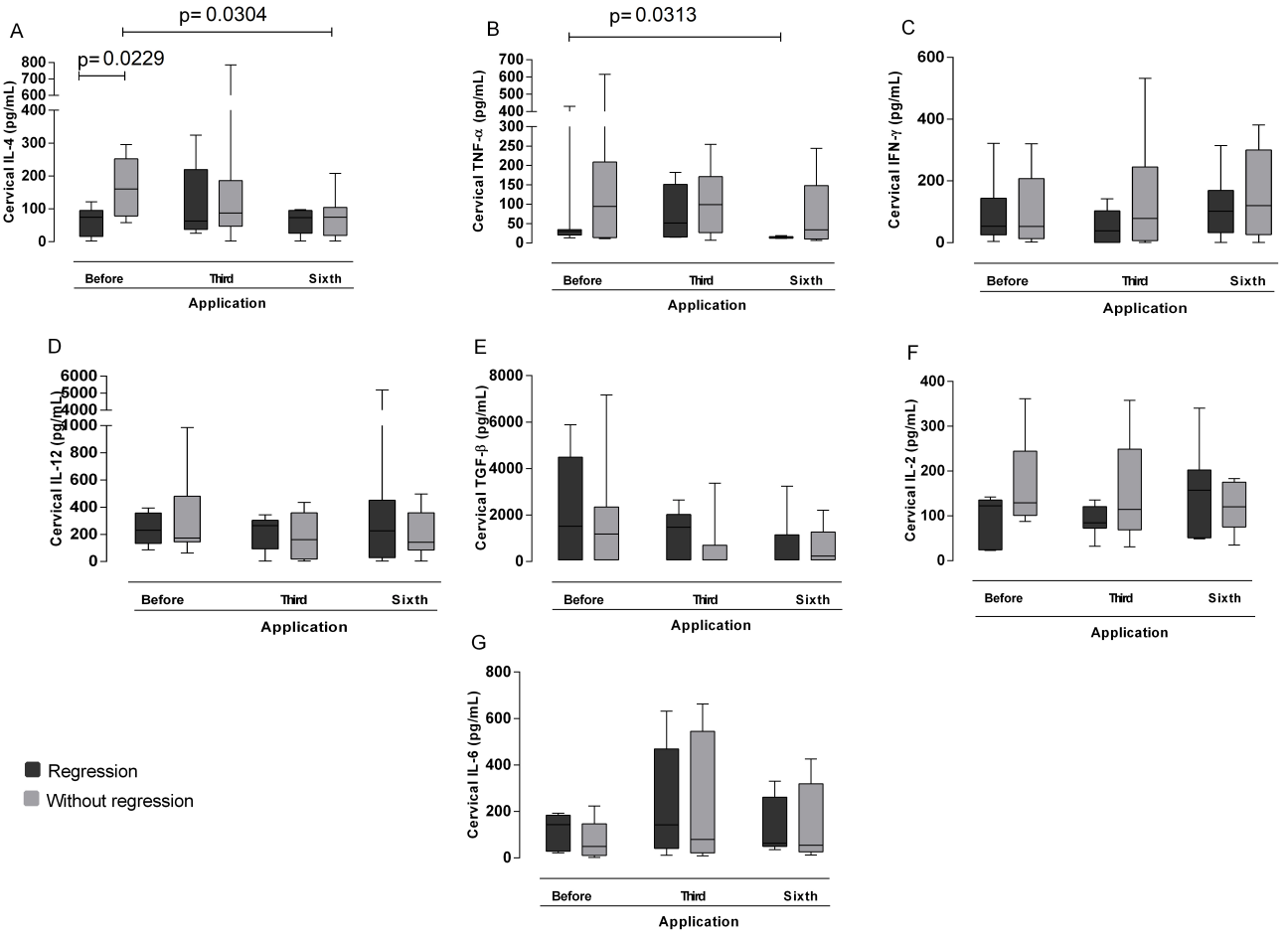

Fig. 3. Concentration of cytokines (IL-2, IL-12, IFN-γ, IL-6, TNF-α TGF-β and IL-4) in endocervical secretion (cervical) of patients with CIN II and III during treatment with IFN-alpha. Dosage performed by ELISA technique and statistical analysis by the Mann Whitney test or Friedman test.

placed in an microtube containing 0.5 mL of 0.9% saline (SF) and its stem was cut so that the tube could be closed. After that, the microtubes containing the brush with the secretion were shaken in a vortex for 1 minute and a hole was made in the lower part. Carefully, the microtube was embedded within another microtube tube (with 0.9% SF) and these were subject to centrifugation for 5 minutes at 300 rpm. After centrifugation, the upper microtube was discarded and the volume of secretion plus saline contained in the (lower) microtube were adjusted to 500 μL with 0.9% SF and the material was stored at -20 °C for cytokine level measurements.

2.3 Serum collection

Venous blood samples were collected before the first application of PEG-Interferon-α-2b, after the third and sixth applications through the vacuum system tubes with separator gel (BD Vacutainer®). After 30 min of coagulation, samples were centrifuged in a refrigerated centrifuge at 4 °C for 10 min at 2000 rpm. Samples were stored in aliquots of 250 μL at -80 °C until use. Serum levels of IL-2, IL-12, IFN-γ, TNF-α, TGF-β, IL-4, IL-6 were determined by ELISA.

2.4 Administration of PEG-Interferon-α-2b

Human recombinant PEG-Interferon-α-2b (Pegintron®; 80 mgc) was injected subcutaneously to the abdominal region at a dose of 80 mgc (flask-ampoule with lyophilic powder diluted in 0.7 mL of diluent before each application). Six injections were performed throughout the treatment, with one injection per week. Peripheral blood was collected of each patient prior the first injection (pretreatment) and after 3th and 6th application.

2.5 Quantification of cytokines by ELISA

The cytokines present in the endocervical secretion and serum were measured by ELISA (Enzyme Linked Immuno Sorbent Assay) with monoclonal antibodies commercially available from BD OptEIA^{TM}. The plates used for analysis contained 384 flats bottom wells and were coated (sensitized) with specific monoclonal antibodies to capture each cytokine. The wells corresponding to the non-reaction did not contain any of the antigens (cytokines) analyzed.

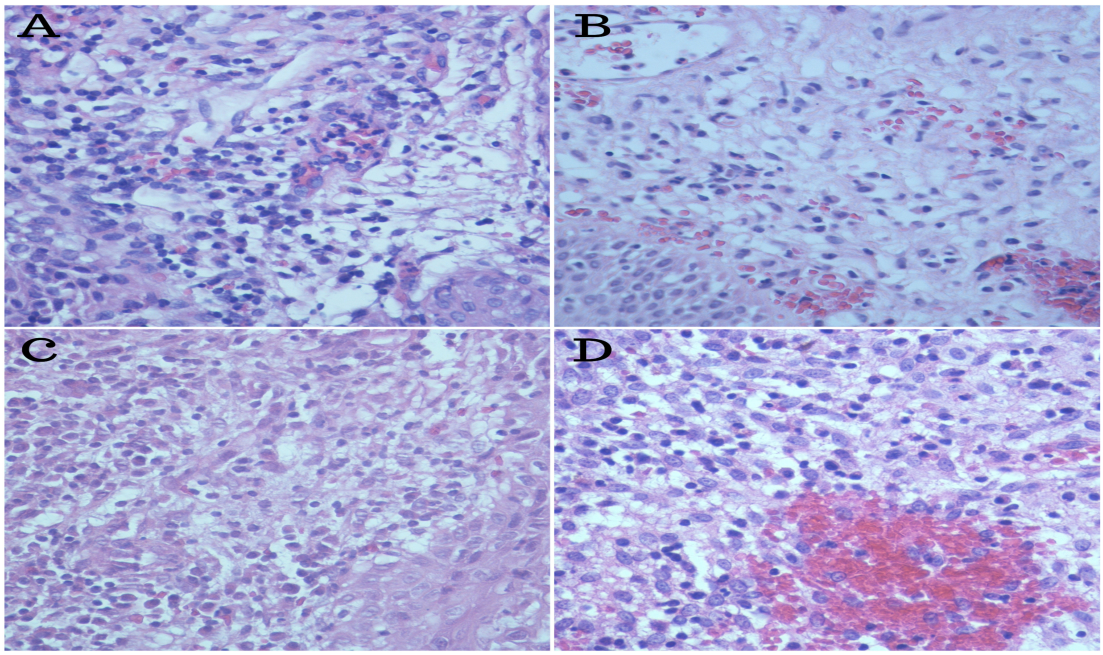

Fig. 4. Inflammatory infiltrate in the stroma underlying the dysplastic areas, predominantly mononuclear composed of lymphocytes and plasmocytes with varying intensity between mild to intense, in all figures (Patient with lesion regression, before (A) and after (B) treatment with IFN-alpha; Patient without regression of lesion before (C) and after (D) treatment with IFN-alpha).

The cytokines, IL-2, IL-12, IFN-γ, TNF-α, TGF-β, IL-4, IL-6 were quantified by ELISA in all secretions selected for the study, following the manufacturer's instructions (BD OptEIA^{TM}). Briefly, for each cytokine quantification, 384-well ELISA microplates (Nunc MaxiSorp®) were coated with monoclonal antibodies, 1 : 250 (BD Biosciences). After, the wells were blocked 50 μL / well with PBS / FBS (10% Fetal Bovine Serum). The standard secretions and solutions were added at 25 μL / well and incubated for 2 hours at room temperature. 25 μL / well of biotin / Streptavidin-HRP (BD Biosciences) conjugated detection monoclonal antibodies, diluted for each cytokine, were added. Then the TMB substrate (BD Biosciences) was used for the development (25 μL/ well) of the assay, read on an automatic ELISA microplate reader (Spectramax 384 Plus) at 450-570 ηm. Concentrations were determined by simple regression on standard curve and values were expressed in ρg /mL.

2.6 Statistical analysis

The levels of the cytokines obtained in endocervical secretions and peripheral blood were evaluated statistically through the program GraphPad Prism 6.0 and Microsoft Excel.

After the normality test, the cytokine levels at the moments of the injections in the same group were compared using the Friedman test, and between the different groups by the Mann-Whitney test. The results were express in median, minimum and maximum values.

3. Results

According to a survey carried out of each patient's medical records, such as histopathology (biopsy prior to treatment and after treatment) and clinical practice, it was observed that 18.75% (n = 3) of these patients presented CIN II) and 81.25% (n = 13) with CIN III at the beginning of treatment. Out of this total, 43.75% (n = 7) had lesion regression after PEG-Interferon-α-2b therapy and 56.25% (n = 9) did not.

During the study, two patients (12.5%) reported some of the symptoms of malaise, lethargy, feverishness, nausea and vomiting. There were no medication-related major complications.

In a survey using medical records of patients who presented lesion regression after treatment with interferon, it was found that only one of the patients required intervention by conization with free margins, 11 months after treatment with interferon, due to the recurrence of CIN III. The other patients have not recur, with cytological control every months.

The general clinical characteristics and the conventional risk factors of the study groups are present in Table 1.

In Fig. 1, the representation by Heat map, where it is possible to visualize the differences of the concentrations of each cytokine by ELISA, in both environments, local and systemic.

Fig. 2 (A-G) shows the cytokine concentration by ELISA (IL-4, TGF-β, TNF-α, IL-6, IL-2, IFN-γ, IL-12) in peripheral blood of patients with CIN II and III treated with PEG-Interferon-α-2b and the Fig. 3 (A-G) demonstrates this concentration in the endocervical secretion of the patients.

Table 1. General clinical features and conventional risk factors of study groups.

Clinical data	Patients with lesion regression (n = 7)	Patients without lesion regression (n = 9)
Age (years)	38 (24-54)	35 (18-82)
Age at 1st intercourse (years)	17 (15-30)	17 (13-19)
Number of sexual partners	3 (1-9)	2 (1-7)
Age at first gestation (years)	27 (17-35)	19 (15-33)
Number of pregnancies	1 (0-5)	2 (0-7)
Number of births	1 (0-4)	2 (0-6)
Number of abortions	0 (0-1)	0 (0-1)

Data collected from the study of patients' medical records. Representation through median (minimum-maximum).

When we analyzed the pre-therapy of responsive and non-responsive patients locally, we observed a higher concentration of IL-4 in patients who did not respond to treatment (P = 0.0229). After treatment, there was a significant reduction in IL-4 in unresponsive patients (P = 0.0304) (Fig. 3A). The profile of local production of TNF-α demonstrated that patients responsive to IFN-α had a reduction in the concentrations of this cytokine after treatment (P = 0.0313) (Fig. 3B).

In the biopsies of these patients there is a predominance of squamous metaplasia, chronic cervicitis. In addition, periodic cytological examinations showed only benign reactive or repairing cellular changes (inflammation), without neoplasia. The inflammatory infiltrate in the stroma underlying the dysplastic areas was evaluated, which in both groups was predominantly mononuclear composed of lymphocytes and plasma cells with varying intensity between mild to intense, apparently with no morphological difference between groups (Fig. 4A,B,C,D).

All patients in question remain under cytological and colposcopic follow-up at the Gynecology and Obstetrics Service of the Federal University of Triângulo Mineiro.

4. Discussion

HPV types 16 and 18 together cause 70-75% of all cervical cancers and 40-60% of their precursor lesions [4].

A study showed that there is an epidemiological association of HPV with an increased risk of HIV infection through differences in cytokine concentrations in the female genital tract, according to HPV status [31].

Alternative treatments such as interferon immunotherapy are described as providing great advantages, such as preservation of the uterine cervix and the reproductive future of patients with high-grade lesions [32], as well in treatment of other diseases such as leukemia and renal carcinomas [33, 34].

In this context, other studies have used interferon as treatment of diseases showing different results, such as regression or persistence of neoplasia [27, 28]. However, in the literature there is no description of the use of PEG-Interferon-α-2b in the treatment of gynecological neoplasia.

In this study, we quantified cytokine levels in order to characterize and understand the profile of local (endocervical secretion) and systemic (peripheral blood) immune response aiming, in the near future, to serve as a protocol for treatment with PEG-Interferon-α-2b of these lesions, reducing conventional medical interventions and preserving the reproductive system, especially in younger patients.

Our study found statistical differences in cytokines IL-4 and TNF-α in endocervical secretion. The other cytokines evaluated did not show statistically significant differences.

Local concentrations of IL-4 demonstrated that patients who did not achieve lesion regression had higher concentrations of this cytokine than patients who achieved lesion regression prior to treatment. After treatment with PEG-Interferon-α-2b, there was a local decrease in IL-4 concentrations in these patients, demonstrating that the treatment can modulate the immune response profile through the production of cytokines. The group without lesion regression already exhibited higher concentrations of IL-4 before treatment with IFN when compared to the group with regression of the lesion. In contrast, the lesion regression group maintained low IL-4 concentrations.

These data suggest that this could be a predominant pattern of response, where several studies have shown that during the carcinogenesis of the cervical epithelium there is a change in Th1 response to Th2 [35, 36]. This downregulation of cellular immunity could explain the immunological loss of control of HPV and its oncological complications [13, 24]. In addition, previous studies have shown that the cervical secretion of patients with HPV infection had increased Th2-profile cytokines, thus, our results are in agreement with reports in literature [37, 38].

Mardegan *et al.* (2011) [25], evaluating patients with high-grade lesions treated with intralesional Interferon-α also observed low levels of this interleukin in their experiments. The expression mRNA of IL-4 in patients with failed therapy suggests that the Th2 immune response pattern would already be present and may have contributed to the persistence of the lesion and to the avoidance of HPV to immunological surveillance [28].

Another cytokine that has an important role in inflammatory reactions inducing secretion of cytokines by several cells of innate and adaptive immunity is the TNF-α, besides inhibiting the expression of HPV E6 and E7 genes [39]. We found in our study a decrease in TNF-α concentration after the last application of IFN-α in the group with lesion regres-

sion, in endocervical secretion. Patients with lesion regression already had lower levels of this cytokine prior to treatment.

The literature shows that one of the major cytokines produced in the differentiation of T helper lymphocytes into the Th2 profile is IL-4, and this profile can inhibit the activation of macrophages, cytotoxic T lymphocytes and suppress the Th1 cell mediated immunity [40].

The absence of TNF-α mRNA prior to intralesional interferon treatment was observed in previous studies, but in our study, cytokine levels remained low at the end of therapy [28]. By this same reasoning, Mardegan *et al.* (2011) [25] also found low levels of this cytokine in vaginal secretions reinforcing the idea that the Th1 profile decreases in high-grade lesions.

A study by Gaiotti *et al.* (2000) [41] associated TNF-α with the activation of RAS and promoting RNA expression of E6 and E7 oncoproteins, thus stimulating the growth of immortalized keratinocytes. This fact may explain low levels of this cytokine in those patients with good therapeutic response. In addition, high levels of TNF-α and TNFR gene variations were associated with CIN [35, 42].

The presence of high levels of TNF-α in the cervix of patients with neoplasia without interferon treatment were significantly elevated in cervical cancer patients compared to precursor lesions, thus demonstrating a change in the local cervical immune environment of patients with cervical cancer [43]. Our study suggests that immunotherapy decreases TNF-α level during treatment.

Knowing that there is an association between chronic inflammation, persistent infection and cancer and that this inflammation can increase up to twice the risk of intraepithelial neoplasia [44], the local decrease in TNF-α might be somehow benefiting patients who responded to treatment with PEG-Interferon-α-2b.

Conservative treatment with IFN-α for patients with CIN II / III has the advantage of preserving reproductive capacity. A study performed by Ramos *et al.* (2010) [28] demonstrated that patients with a satisfactory response (60%) to IFN-alpha-2b treatment expressed more Th1 (IFN-γ, TNF-α, IL-2) cytokines with a significant reduction in high-risk HPV viral load. Patients with failed therapy were smokers and had higher expression of Th2 (IL-4) or Treg (TGF-beta2 and TGF-beta3) cytokines. Our study demonstrated that treatment with IFN-α induces the reduction of IL-4, not being, in this case, the consequence of the increase of IL-12.

A change in the secretion profile of Th1 to Th2 cytokines, with the loss of cellular immunity, can lead to cervical cancer and high-grade lesions in HPV-infected women, as well as to promote a persistent infection, enabling a greater likelihood of HPV carcinogenesis [45]. The decrease in Th1 response and increase in Th2 are associated with the development of cervical cancer [46].

Of the 16 patients studied, 7 patients (43.75%) showed regression of the lesion after treatment with PEG-Interferon-α-2b and 9 (56.25%) did not. The success rate in the regression of the lesion is in agreement with other studies that have used Interferon-α as a form of treatment, since the percentage of cure or stabilization of the neoplasia ranges from 33% to 90%, according to literature [28, 47].

Study suggest that cervical cancer may be a good target for immunotherapy, also in the neoadjuvant setting, with the expression of PD-L1 was significantly associated with stromal TILs [48].

We are aware of the small number of patients, and the few cycles of therapy, but our preliminary findings encourages us to continue our studies with immunotherapies.

5. Conclusions

Our findings allow us to conclude that CIN regression is related to the immune profile, mainly local, before treatment, as in the case of IL-4, promoting a Th2 profile in those patients with therapeutic failure. These reinforces the need for an in-depth analysis of immunological dysregulation in these patients. Assessing the cervical immune profile can provide information that could predict the patient's response to treatment.

Possibly, from a clinical point of view, regression of CIN III to CIN II does not present great effect, but immunologically we can perceive progresses caused by treatment with the PEG-Interferon-α-2b.

Perhaps in the near future other questions can be elucidated as to the treatment and response of HPV-induced lesions so that, even in the absence of regression, such therapy may at least reduce neoplasia, making conventional medical interventions less aggressive, thus preserving the reproductive potential of young patients.

Author contributions

Márcia Antoniazi Michelin and Eddie Fernando Candido Murta conceived and designed the experiments; Fabiano Vilela Mundim and Marco Aurélio Trovó performed the experiments; Fabiano Vilela Mundim, Letícia Montes Stark and Millena Prata Jammal analyzed the data; Fabiano Vilela Mundim, Letícia Montes Stark and Millena Prata Jammal wrote the paper.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Research Ethics Committee of the institution (CEP / UFTM nº 759 and 1525).

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Conflict of interest

The authors declare no conflict of interest.

References

[1] Burd EM. Human papillomavirus and cervical cancer. Clinical Microbiology Reviews. 2003; 16: 1-17.

[2] Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, MacIntyre MF, *et al.* The global burden of cancer 2013. JAMA Oncology. 2016; 1: 505-527.

[3] Muñoz N, Castellsagué X, de González AB, Gissmann L. Chapter 1: HPV in the etiology of human cancer. Vaccine. 2006; 24: S1-S10.

[4] Arbyn M, Weiderpass E, Bruni L, de Sanjosé S, Saraiya M, Ferlay J, *et al.* Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. The Lancet Global Health. 2020; 8: e191-e203.

[5] Frazer IH. Interaction of human papillomaviruses with the host immune system: a well evolved relationship. Virology. 2009; 384: 410-414.

[6] Lucs AV, DeVoti JA, Hatam L, Afzal A, Abramson AL, Steinberg BM, *et al.* Immune dysregulation in patients persistently infected with human papillomaviruses 6 and 11. Journal of Clinical Medicine. 2015; 4: 375-388.

[7] Téllez L, Michelli E, Mendoza JA, Vielma S, Noguera M, Callejas D, *et al.* Persistent infection with high-risk human papilloma viruses: cohort study, Mérida, Venezuela. Ecancermedicalscience. 2015; 9: 579.

[8] van der Weele P, van Logchem E, Wolffs P, van den Broek I, Feltkamp M, de Melker H, *et al.* Correlation between viral load, multiplicity of infection, and persistence of HPV16 and HPV18 infection in a Dutch cohort of young women. Journal of Clinical Virology. 2016; 83: 6-11.

[9] Wang J, Elfström KM, Andrae B, Nordqvist Kleppe S, Ploner A, Lei J, *et al.* Cervical cancer case-control audit: results from routine evaluation of a nationwide cervical screening program. International Journal of Cancer. 2020; 146: 1230-1240.

[10] Lo Cigno I, Calati F, Albertini S, Gariglio M. Subversion of host innate immunity by human papillomavirus oncoproteins. Pathogens. 2020; 9: 292.

[11] Gutiérrez-Hoya A, Soto-Cruz I. Role of the JAK/STAT pathway in cervical cancer: its relationship with HPV E6/E7 oncoproteins. Cells. 2020; 9: 2297.

[12] Geginat J, Paroni M, Maglie S, Alfen JS, Kastirr I, Gruarin P, *et al.* Plasticity of human CD4 T cell subsets. Frontiers in Immunology. 2014; 5: 630.

[13] Agarossi A, Casolati E, Valieri M, Ferrazzi E, Maffeis G, Trabattoni D, *et al.* Mucosal immune response to Human Papilloma Virus (HPV) infection in HIV positive women. Medycyna Wieku Rozwojowego. 2004; 7: 495-502.

[14] Sharma A, Rajappa M, Saxena A, Sharma M. Cytokine profile in Indian women with cervical intraepithelial neoplasia and cancer cervix. International Journal of Gynecological Cancer. 2007; 17: 879-885.

[15] Fernandes APM, Gonçalves MAG, Duarte G, Cunha FQ, Simões RT, Donadi EA. HPV16, HPV18, and HIV infection may influence cervical cytokine intralesional levels. Virology. 2005; 334: 294-298.

[16] Gibbert K, Schlaak JF, Yang D, Dittmer U. IFN-α subtypes: distinct biological activities in anti-viral therapy. British Journal of Pharmacology. 2013; 168: 1048-1058.

[17] Isaacs A, Lindenmann J. Virus interference. I. The interferon. Proceedings of the Royal Society of London Series B, Biological Sciences. 1957; 147: 258-267.

[18] Machado PRL, Araújo MIAS, Carvalho LC, Edgar M. Mecanismos de resposta imune à s alterações. Anais Brasileiros de Dermatologia. 2004; 79: 647-662. (In Portuguese)

[19] Parmar S, Platanias LC. Interferons: mechanisms of action and clinical applications. Current Opinion in Oncology. 2003; 15: 431-439.

[20] Apgar BS, Wright TC, Pfenninger JL. Loop electrosurgical excision procedure for CIN. American Family Physician. 1992; 46: 505-520.

[21] Barbero M, Gallia L, Canni M, Bocci F, Bertini U, Ghiringhello B, *et al.* Treatment of cervical intraepitelial neoplasia using the loop electrosurgical excision procedure. Tumori. 2002; 87: S12-S13.

[22] Hillemanns P, Kimmig R, Dannecker C, Noorzai T, Diebold J, Thaler CJ, *et al.* Schlingenkonisation versus Messerkonisation zur Therapie zervikaler intraepithelialer Neoplasien [LEEP versus cold knife conization for treatment of cervical intraepithelial neoplasias]. Zentralblatt Fur Gynakologie. 2000; 122: 35-42. (In German)

[23] Clerici M, Lucey D, Berzofsky J, Pinto L, Wynn T, Blatt S, *et al.* Restoration of HIV-specific cell-mediated immune responses by interleukin-12 *in vitro*. Science. 1993; 262: 1721-1724.

[24] de Jong A, van Poelgeest MIE, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJM, *et al.* Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Research. 2004; 64: 5449-5455.

[25] Mardegan MC, Ramos MC, Adad SJ, Michelin MA, Shimba D, Murta EFC. Immunological evaluation of vaginal secretion in patients with high-grade cervical intraepithelial neoplasia treated with intralesional interferon alpha-2b. European Journal of Gynaecological Oncology. 2011; 32: 297-302.

[26] Michelin MA, Montes L, Nomelini RS, Trovó MA, Murta EFC. Helper T lymphocyte response in the peripheral blood of patients with intraepithelial neoplasia submitted to immunotherapy with pegylated interferon-α. International Journal of Molecular Sciences. 2015; 16: 5497-5509.

[27] Misson DR, Abdalla DR, Borges AM, Shimba DS, Adad SJ, Michelin MA, *et al.* Cytokine serum levels in patients with cervical intraepithelial neoplasia grade II-III treated with intralesional interferon-α 2b. Tumori Journal. 2011; 97: 578-584.

[28] Ramos MC, Mardegan MC, Peghini BC, Adad SJ, Michelin MA, Murta EFC. Expression of cytokines in cervical stroma in patients with high-grade cervical intraepithelial neoplasia after treatment with intralesional interferon alpha-2b. European Journal of Gynaecological Oncology. 2010; 31: 522-529.

[29] Sikorski M, Bobek M, Zrubek H, Marcinkiewicz J. Dynamics of selected MHC class I and II molecule expression in the course of HPV positive CIN treatment with the use of human recombinant IFN-gamma. Acta Obstetricia et Gynecologica Scandinavica. 2004; 83: 299-307.

[30] Montes L, Andrade CM, Michelin MA, Murta EF. The importance of alpha/beta (alpha/13) interferon receptors and signaling pathways for the treatment of cervical intraepithelial neoplasias. European Journal of Gynaecological Oncology. 2014; 35: 368-372.

[31] Liebenberg LJP, McKinnon LR, Yende-Zuma N, Garrett N, Baxter C, Kharsany ABM, *et al.* HPV infection and the genital cytokine milieu in women at high risk of HIV acquisition. Nature Communications. 2019; 10: 5227.

[32] Murta EFC, Tavares Murta BM. Successful pregnancy after vaginal cancer treated with interferon. Tumori. 2004; 90: 247-248.

[33] Bex A, Mallo H, Kerst M, Haanen J, Horenblas S, de Gast GC. A phase-II study of pegylated interferon alfa-2b for patients with metastatic renal cell carcinoma and removal of the primary tumor. Cancer Immunology, Immunotherapy: 2005; 54: 713-719.

[34] George S, Hutson TE, Mekhail T, Wood L, Finke J, Elson P, *et al.* Phase i trial of PEG-interferon and recombinant IL-2 in patients with metastatic renal cell carcinoma. Cancer Chemotherapy and Pharmacology. 2008; 62: 347-354.

[35] Azar KK, Tani M, Yasuda H, Sakai A, Inoue M, Sasagawa T. Increased secretion patterns of interleukin-10 and tumor necrosis factor-alpha in cervical squamous intraepithelial lesions. Human Pathology. 2004; 35: 1376-1384.

[36] Delgado FG, Martínez E, Céspedes MA, Bravo MM, Navas MC, Cómbita Rojas AL. Increase of human papillomavirus-16 E7-specific T helper type 1 response in peripheral blood of cervical cancer patients after radiotherapy. Immunology. 2009; 126: 523-534.

[37] Bais AG, Beckmann I, Ewing PC, Eijkemans MJC, Meijer CJLM, Snijders PJF, *et al.* Cytokine release in HR-HPV (+) women without and with cervical dysplasia (CIN II and III) or carcinoma, compared with HR-HPV (-) controls. Mediators of Inflammation. 2007; 2007: 24147.

[38] Tjiong MY, Out TA, Schegget JT, Burger MPM, Van Der Vange N. Epidemiologic and mucosal immunologic aspects of HPV infection and HPV-related cervical neoplasia in the lower female genital tract: a review. International Journal of Gynecological Cancer. 2001; 11: 9-17.

[39] Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nature Reviews Immunology. 2003; 3: 745-756.

[40] Pardo-Govea T, Callejas D, Núñez-Troconis J, Araujo M, Costa L, Pons H, *et al.* Expresión de interferon gamma (IFN-gamma), factor de necrosis tumoral alfa (TNF-alpha) e interleucinas 2, 4 y 6 (IL-2, IL-4, IL-6) en células de neoplasias intraepiteliales de cuello uterino: reporte preliminar [Gamma interferon (IFN-gamma), tumor necrosis factor alpha (TNF-alpha) and interleukins 2, 4 and 6 (IL-2, IL-4, IL-6) in cervical-uterine cells of intraepithelial neoplasia: a preliminary report]. Investigacion Clinica. 2005; 46: 5-13.

[41] Gaiotti D, Chung J, Iglesias M, Nees M, Baker PD, Evans CH, *et al.* Tumor necrosis factor-alpha promotes human papillomavirus (HPV) E6/E7 RNA expression and cyclin-dependent kinase activity in HPV-immortalized keratinocytes by a ras-dependent pathway. Molecular Carcinogenesis. 2000; 27: 97-109.

[42] Singhal P, Sharma U, Hussain S, Nag A, Bharadwaj M. Identification of genetic variants in TNF receptor 2 which are associated with the development of cervical carcinoma. Biomarkers. 2016; 21: 665-672.

[43] Tjiong MY, Van Der Vange N, ter Schegget JS, Burger MP, ten Kate FW, Out TA. Cytokines in cervicovaginal washing fluid from patients with cervical neoplasia. Cytokine. 2001; 14: 357-360.

[44] Fernandes JV, De Medeiros Fernandes TAA, De Azevedo JCV, Cobucci RNO, De Carvalho MGF, Andrade VS, *et al.* Link between chronic inflammation and human papillomavirus-induced carcinogenesis (Review). Oncology Letters. 2015; 9: 1015-1026.

[45] Bais AG, Beckmann I, Lindemans J, Ewing PC, Meijer CJ, Snijders PJ, Helmerhorst TJ. A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions. Journal of Clinical Pathology. 2005; 58: 1096-10100.

[46] Scott M, Nakagawa M, Moscicki A. Cell-mediated immune response to human papillomavirus infection. Clinical Diagnostic Laboratory Immunology. 2001; 8: 209-220.

[47] Machado FA, Abdalla DR, Montes L, Etchebehere RM, Michelin MA, Murta EFC. An evaluation of immune system cell infiltrate in the cervical stroma of patients with grade III cervical intraepithelial neoplasia after treatment with intralesional alpha-2B interferon. European Journal of Gynaecological Oncology. 2014; 35: 20-25.

[48] D'Alessandris N, Palaia I, Pernazza A, Tomao F, Di Pinto A, Musacchio L, *et al.* PD-L1 expression is associated with tumor infiltrating lymphocytes that predict response to NACT in squamous cell cervical cancer. Virchows Archiv. 2020.