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Immunohistochemical analysis of CD68, CD4, CD8 and CD20 expression in cervical dysplasia and its relationship with HR-HPV infection

J. RAONIC[1], M. LOPICIC[2], L. VUCKOVIC[1], J. VUCINIC[1]

[1]Clinical Centre of Montenegro, Centre for Pathology, University of Montenegro, Faculty of Medicine, Podgorica, Montenegro
[2]Institute for Public Health, Centre for Medical Microbiology, Podgorica, Montenegro

Abstract. – OBJECTIVE: The aim of the study was to examine the composition of the inflammatory infiltrates in cervical premalignant lesions and contribute to a better understanding of immune response to HR-HPV infection and dysplasia.

PATIENTS AND METHODS: Semi-quantitative analysis of CD68, CD4, CD8 and CD20 immunohistochemical expression in a series of 41 cervical biopsies without dysplasia, 24 cases of LSIL and 35 HSIL cases was performed. In each subject, genotyping for 12 HR-HPV types was done prior to the biopsy.

RESULTS: Observing the total sample, no correlation between CD68, CD4, CD8 and CD20 expression levels and HR-HPV infection was found, regardless of the presence of mono- or co-infection ($p>0.05$). A statistically significant correlation between dysplastic changes and CD68 expression, as well as between dysplastic changes and CD4 expression, was observed ($p=0.003$ and $p=0.016$, respectively). For CD68 expression, there was a positive correlation with both LSIL and HSIL, and concerning CD4 expression, there was a positive correlation primarily with LSIL. The finding of mild CD68 expression shows a 10.5 times greater chance of the sample being classified as LSIL, while the finding of a strong CD68 expression shows a 12 times greater chance of the sample being classified as HSIL, in comparison to cases with no expression. When the samples were stratified in relation to the lesion grade, a correlation between HR-HPV infection and CD68/CD4 expression again was not proved ($p>0.05$). No correlation between CD8 and CD20 expression with dysplasia was found ($p>0.05$).

CONCLUSIONS: We consider a higher prevalence of macrophages and CD4 lymphocytes in dysplastic lesions to be a response to dysplasia rather than HR-HPV infection itself. The increase of the expression levels of macrophages with the degree of the lesion speaks in favour of their potential role in the progression of the neoplastic process.

Key Words:
HR-HPV, Cervical dysplasia, Inflammation.

Introduction

Cervical carcinoma is still a significant cause of morbidity and mortality, despite the efforts put into its suppression. It represents the fourth most frequent malignity among the female population[1]. It is estimated that in 2020, 604,127 women were diagnosed with cervical carcinoma worldwide[2].

Almost all cervical carcinoma cases are related to persistent high-risk human papillomavirus (HR-HPV) infection[3,4]. Dysplastic changes in the cervical epithelia – low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesions (HSIL) – precede the development of cancer.

It is assumed that most women get genital HPV infection during their life, with a prevalence of 50-80%[5]. In most of those cases an HPV infection, as well as LSIL resolution, occurs as a result of the cellular immune response action, which is usually, but not necessarily, followed by seroconversion and antibody production[6].

The female reproductive tract contains all the necessary elements for an effective immune response to genital pathogens and it is estimated that white blood cells make up a significant part of the cellular population of the female genital tract[7,8]. However, around 10-15% of women do not achieve a successful immune response and remain HPV-positive with a constant virus production[9,10] and those women are at risk of HSIL and the development of cervical carcinoma.

The two most significant oncoproteins in HPV-induced carcinogenesis are E6 and E7. E6 protein bonds a p53 tumour suppressor protein

Corresponding Author: Janja Raonic, MD; e-mail: janja@t-com.me

and induces its degradation *via* a ubiquitin-mediated process, while E7 protein bonds cyclin-2 ubiquitin ligase complex and ubiquitinates retinoblastoma tumour suppressor protein. Apart from this, E7 protein also inactivates the inhibitors of the cyclin-dependent kinases CDKN1A (p21) and CDKN1B (p27), and possibly activates cyclins E and A[11,12].

The overall role of immune control in carcinogenesis is a frequent subject of scientific studies. According to Hanahan and Weinberg[13], adequate immune control, primarily through T-lymphocyte function and integrity preservation, enables the detection and elimination of malignantly transformed cells even before the disease becomes clinically evident. Even though the tumour cells develop different mechanisms for bypassing the immune control of the organism, the results of numerous studies on different solid tumours point to the existence of a strong connection between a larger number of tumour-infiltrating lymphocytes in the stroma and better prognosis of the tumour itself[14].

In this sense, apart from the recognised significance of studying the composition of the intratumoural inflammatory infiltrate, quantitative analysis of this infiltrate in precancerous lesions, for which the cervix is an ideal candidate, could make a significant contribution to understanding the evolution and complexity of the immune response to dysplasia and malignancy. A literature review regarding the presence of certain inflammatory cells in the inflammatory infiltrate in premalignant and malignant cervical lesions and their connection with HPV infection and the neoplastic process shows conflicting results.

In our study, we analysed the composition of the inflammatory infiltrate through a semi-quantitative assessment of macrophages, the CD4 and CD8 subpopulations of T lymphocytes and B lymphocytes in early and late cervical dysplastic lesions and we also assessed their presence in relation to the presence/absence of HR-HPV infection.

Patients and Methods

The study was conducted at the Clinical Centre of Montenegro and included 107 voluntary female subjects who had a clinical indication for a cervical biopsy. According to our institution protocols, the indications were an abnormal Pap test and/or an abnormal colposcopy finding. All the women signed informed consent forms. Prior to the biopsy, cervical swabs for HR-HPV detection were taken from each subject.

HR-HPV Sample Collection, DNA Extraction and Genotyping

For HPV testing, cervical swabs were collected using a cytobrush (Kito-Brush, Kaltek, Padova, Italy). The samples were placed in a specimen transport medium – ThinPrep Pap Test PreservCyt® Solution (Cytic Corporation, Marlborough, MA, USA) – in a 20 ml vial and stored at a temperature of -70° C. The DNA extraction and HPV genotyping were carried out at the Centre for Medical Microbiology, Institute of Public Health of Montenegro.

After dissolving the sample, the solution was vortexed and then 1-10 ml of each sample (5 ml clear; 3 ml cloudy) was transferred to a sterile 1.5 ml plastic tube and centrifuged at 1300 rpm for 12 minutes. The supernatant was dried with a Pasteur pipette (3 ml), and the precipitate was used for DNA extraction. The DNA was isolated using the DNA-Sorb-A extraction kit according to the manufacturer's instructions (REF K-1-1/A, Sacace Biotechnologies, Como, Italy).

The detection and genotyping of the HPV DNA were performed by an HPV High-Risk Typing Real-TM test (Sacace Biotechnologies, Como, Italy) used for qualitative detection and genotyping of 12 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59). The test was based on multiplex real-time PCR amplification run in four tubes for each sample. Each tube contained primers directed against regions of three HPV types with the human β-globin gene used as the internal control. A total of 20 µl of nucleic acid extracts per sample were used in four PCR reactions (the 8 µl master mix and 5 µl eluate made up 13 µl of each of the four PCR mixes). Regarding the validity of the test used, the authors relied on the certificate provided by the manufacturer.

Processing and Analysis of Biopsy Specimens

The biopsy samples were paraffin-embedded, stained with the standard haematoxylin and eosin technique, and then, analysed by two independent pathologists with no prior knowledge of the patients' clinical data, nor of their HPV status. In six cases, the biopsy samples were unsuitable for further immunohistochemical analysis (scarce or crushed artefact) and only one case showed the presence of developed cervical

carcinoma, therefore, these samples were dismissed. The other 100 samples were classified into three groups: without dysplasia (41), LSIL (24) and HSIL (35).

The selected tissue sections were treated in a 10 mM citrate buffer in a microwave oven two times for 10 minutes, and then, washed out with deionised water. After the deparaffinisation and antigen demasking procedure, the endogen peroxidase was blocked using a 3% H_2O_2 solution for 10 minutes at room temperature. The tissue sections were then incubated with the primary antibody in a moist chamber for 1 hour at room temperature. Immunohistochemical identification of the tested antigens was performed by the streptavidin-biotin-peroxidase technique according to the standard LSAB+ procedure (DAKO, Carpinteria, CA and Glostrup, Denmark). The sections were first incubated with biotinylated anti-mouse antibody for 30 minutes at room temperature, and then, by a streptavidin-peroxidase complex for another 30 minutes. As a chromogen substrate, 3-amino-9-ethylcarbazole (AEC, DAKO, Carpinteria, CA and Glostrup, Denmark) was applied. After each incubation, the samples were washed out in Tris-buffered saline (TBS: 0.05 M, pH 7.6) and contrasted by haematoxylin. The sections were covered by a special water medium. The following primary antibodies were used: CD68 (Monoclonal Mouse Anti-Human CD68, Clone PG-M1, FLEX Ready to use, DAKO, Carpinteria, CA and Glostrup, Denmark); CD4 (Monoclonal Mouse Anti-Human CD4, Clone 4B12, FLEX Ready to use, DAKO, Carpinteria, CA and Glostrup, Denmark); CD8 (Monoclonal Mouse Anti-Human CD8, Clone C8/144B, FLEX Ready to use, DAKO, Carpinteria, CA and Glostrup, Denmark); CD20 (Monoclonal Mouse Anti-Human CD20cy, Clone L26, FLEX Ready to use, DAKO, Carpinteria, CA and Glostrup, Denmark).

An evaluation of the immunoreactivity of the inflammatory infiltrate in the epithelium and stroma just beneath the epithelium was first performed by analysing the slides at 100× magnification. Further analysis included the five fields with the greatest number of immunoreactive cells – hot spots. At 400× magnification of the five selected fields and on the basis of the middle value of expression, semi-quantitative scoring was carried out: 0 (without expression) – <5% immunoreactive cells; 1 (weak expression) – 5-20% immunoreactive cells; 2 (mild expression) – 20-50% immunoreactive cells; 3 (strong expression) – >50% immunoreactive cells.

Statistical Analysis

Data analysis was performed in IBM SPSS Statistics version 23.0 software (IBM SPSS for Windows, Armonk, NY, USA), using both descriptive and inferential statistical methods. Statistical significance was examined using tests for nonparametric data – χ^2 test and Fisher's test. Examination of the predictive value of different risk factors was performed using multinomial logistic regression. For all the statistical analyses, the level of significance was 0.05.

Results

HR-HPV infection was determined in 55 (55%) subjects, 40 (40%) of whom had a mono-infection and 15 (15%) a co-infection with more than one HR-HPV genotype. Observing mono-infections, the most frequent genotypes were: type 16 (16; 25.9%), type 31 (8; 14.8%) and type 45 (6; 11.1%). In cases where co-infection was detected, the most frequent combination of HR-HPV genotypes was 16 and 51 (3; 5.6%), while the combinations 16 and 52, and 16 and 33 were found in two (3.7%) subjects.

No correlation between CD68, CD4, CD8 and CD20 expression levels and the presence of HR-HPV infection was found. The same result was obtained when the expression of these markers was observed in relation to the existence of mono- and co-infection. The immunoreactivity score distribution in HR-HPV+/HR-HPV– cases and in HR-HPV mono- and co-infection cases is summarised in Table I. Using multinomial logistic regression, it was determined that CD68, CD4, CD8 and CD20 do not represent good predictors and that their expression level has no statistically significant connection with the presence of HR-HPV infection or with the presence of monotypic or multiple HR-HPV infection.

The CD68, CD4, CD8 and CD20 immunoreactivity score distribution in relation to the presence/absence of dysplastic changes is shown in Table II. We found a statistically significant correlation between CD68 expression and the presence of dysplastic changes – Fisher's test = 18.345; p=0.003. The value of the gamma correlation coefficient (γ=0.358; p=0.004) shows a moderately strong, positive correlation between CD68 expression and the existence of dysplastic changes, and the results of multinomial logistic regression show a statistically significant connection between CD68 expression and the presence

Table I. CD68, CD4, CD8 and CD20 immunoreactivity score distribution in HR-HPV+/HR-HPV- cases and in HR-HPV mono-infection /co-infection.

	HR-HPV-	HR-HPV+	*p*-value	HR-HPV mono-infection	HR-HPV co-infection	*p*-value
CD68			0.213			0.622
0	10 (10%)	20 (20%)		14 (25.5%)	6 (10.9%)	
1	14 (14%)	16 (16%)		11 (20%)	5 (9.1%)	
2	19 (19%)	14 (14%)		12 (21.8%)	2 (3.6%)	
3	2 (2%)	5 (5%)		3 (5.5%)	2 (3.6%)	
CD4			0.093			0.819
0	40 (40%)	41 (41%)		28 (50.9%)	13 (23.6%)	
1	5 (5%)	7 (7%)		6 (10.9%)	1 (1.8%)	
2	0 (0%)	5 (5%)		4 (7.3%)	1 (1.8%)	
3	0 (0%)	2 (2%)		2 (3.6%)	0 (0%)	
CD8			0.442			0.387
0	31 (31%)	37 (37%)		26 (47.3%)	10 (20%)	
1	11 (11%)	10 (10%)		6 (10.9%)	4 (7.3%)	
2	3 (3%)	5 (5%)		5 (9.1%)	0 (0%)	
3	0 (0%)	3 (3%)		3 (5.5%)	0 (0%)	
CD20			0.156			0.293
0	28 (28%)	27 (27%)		19 (34.5%)	8 (14.5%)	
1	11 (11%)	11 (11%)		6 (10.9%)	5 (9.1%)	
2	4 (4%)	7 (7%)		6 (10.9%)	1 (1.8%)	
3	2 (2%)	10 (10%)		9 (16.4%)	1 (1.8%)	

of LSIL and HSIL changes. The finding of mild CD68 expression shows a 10.5 times greater chance of the sample being classified as LSIL compared to those in which there was no expression of the same marker. The finding of strong CD68 expression shows a 12 times greater chance of the sample being classified as HSIL compared to those in which there was no expression (Table III). CD68 expression in a normal cervix and LSIL and HSIL lesions is shown in Figure 1. Regarding the previous results, we further analysed the level of CD68 expression in HR-HPV+ and HR-HPV– cases with developed dysplastic changes. No correlation between the level of

Table II. Distribution of CD68, CD4, CD8 and CD20 immunoreactivity scores in biopsy samples with and without dysplastic lesions.

	Without dysplasia	LSIL	HSIL	*p*-value
CD68				0.003
0	18 (18%)	3 (3%)	9 (9%)	
1	14 (14%)	7 (7%)	9 (9%)	
2	8 (8%)	14 (14%)	11 (11%)	
3	1 (1%)	0 (0%)	6 (6%)	
CD4				0.016
0	39 (39%)	18 (18%)	24 (24%)	
1	2 (2%)	5 (5%)	5 (5%)	
2	0 (0%)	1 (1%)	4 (4%)	
3	0 (0%)	0 (0%)	2 (2%)	
CD8				0.320
0	0.016	0 (0%)	0 (0%)	
1	31 (31%)	14 (14%)	23 (23%)	
2	7 (7%)	7 (7%)	7 (7%)	
3	3 (3%)	3 (3%)	2 (2%)	
CD20				0.240
0	27 (27%)	13 (13%)	15 (15%)	
1	9 (9%)	6 (6%)	7 (7%)	
2	3 (3%)	3 (3%)	5 (5%)	
3	2 (2%)	2 (2%)	8 (8%)	

Table III. Cox PH regression model estimates for the risk of clinical recurrence.

CD68	LSIL OR (95% CI) *p*	HSIL OR (95% CI) *p*	CD4	LSIL OR (95% CI) *p*
0	1 (reference group)	1 (reference group)	0	1 (reference group)
1	3 (0.66-13.75) $p = 0.157$	1.29 (0.40-4.09) $p = 0.671$	1	4.77 (0.76-29.81) $p = 0.094$
2	10.5 (2.34-47.03) $p = 0.002$	2.75 (0.82-9.24) $p = 0.091$	2	2.45 (1.95-15.52) $p = 0.997$
3	0.05 (0.03-1.14) $p = 0.088$	12 (1.25-115.36) $p = 0.031$	3	1.27 (1.25-2.34) $p = 0.041$

CD68 expression and the presence of HR-HPV infection was found either in samples with LSIL (Fisher's test = 2.537, p=0.055) or in those with HSIL (Fisher's test = 0.004, p=0.677) (Table IV).

Our results show a strong positive correlation between CD4 expression and dysplastic changes (Fisher's test = 12.325; p=0.016), with a gamma correlation coefficient (γ=0.591; p=0.001). Results of multinomial logistic regression showed that CD4 expression had a statistically significant correlation primarily with the existence of LSIL, but not with the existence of HSIL. The finding of strong CD4 expression shows a 1.27 times greater chance of the sample being classified as LSIL compared to those in which there was no expression of this marker (Table III). Similar to the result of CD68 expression, no correlation between the level of CD4 expression in HR-HPV+ and HR-HPV– cases, was found in either LSIL (Fisher's test = 1.233, p=0.458) or HSIL (Fisher's test = 0.934, p=0.454) (Table IV).

We did not find any connection between the levels of CD8 and CD20 expression with the presence/absence of dysplasia. The values of Fisher's test are: 6.575; p=0.320 for CD8; and 7.873; p=0.240 for CD20. The results of multinomial logistic regression showed that these markers do not represent good predictors and that the levels of their expression do not have a statistically significant connection with the existence of dysplastic cervical lesions.

Discussion

Among our study group, a high prevalence of HR-HPV genotypes was found with more than half of the infected women. Interestingly, when observing the overall sample, no correlation between HR-HPV infection and the abundance of macrophages, B lymphocytes, CD4 or CD8 subpopulations of T lymphocytes in inflammatory infiltrates was found, regardless of the presence of monotypic infection or co-infection.

On the other hand, our results showed a correlation between macrophage expression and the presence of dysplastic lesions. Although in some studies the exact opposite results were obtained[15,16], which certain authors explain as a result of a suboptimal selection of antibodies for macrophage visualisation, as well as due to the scope for interpretation[17], the results of a larger

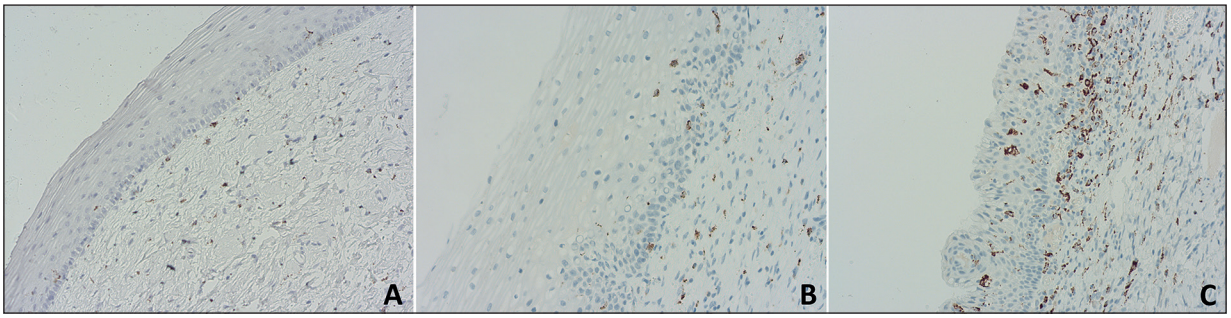

Figure 1. CD68 expression in: (**A)** a normal cervix, ×100 **(B)** LSIL, ×200 **(C)** HSIL, ×100.

Table IV. Distribution of CD68 and CD4 expression in hrHPV+ and hrHPV – cases with dysplasia.

		CD68			CD4		
		Weak expression	Strong expression	*p*-value	Weak expression	Strong expression	*p*-value
LSIL				0.055			0.458
	HR-HPV+	7 (29.2%)	4 (16.7%)		10 (41.7%)	1 (4.2%)	
	HR-HPV-	3 (12.5%)	10 (41.7%)		13 (54.2%)	0 (0%)	
HSIL				0.677			0.454
	HR-HPV+	16 (45.7%)	15 (42.9%)		25 (71.4%)	6 (17.1%)	
	HR-HPV-	2 (5.7%)	2 (5.7%)		4 (11.4%)	0 (0%)	

number of studies match our results, showing a connection between the level of expression of the infiltrating macrophages and the grade of cervical lesions[17,18].

In addition to finding greater macrophage expression in dysplastic lesions, a study conducted by Chen et al[18] also found a correlation between this expression and HR-HPV infection.

In our research, as in the research conducted by Hammes et al[17], as well as in the study of Davidson et al[15], a correlation between HR-HPV infection and macrophage expression stratified in relation to the lesion grade was not proved. In these studies, this finding was not discussed. Regarding our results, we think that macrophage infiltration occurs as a response to the started neoplastic process, which is followed by morphological changes to the cervical epithelium, so that HR-HPV infection itself has no effect on their response.

Taking into account our results, as well as the results of the mentioned studies, which included cases of cervical carcinoma, we believe that macrophages whose number in the infiltrate increases with the lesion grade contribute to the progress of the neoplastic process.

Macrophages are the carriers of innate immunity and, through their ability to process and present antigens, to produce the cytokines necessary for T lymphocyte activation, they are crucial in initiating and mediating a specific immune response[19]. In this context, macrophages play an important role in fighting infection, the resolution of acute inflammation, but also in the regulation of the metabolic response to tissue stress[20]. However, macrophages represent a phenotypically heterogeneous group of cells. Their physiology can be significantly modified in response to various biochemical factors from the microenvironment[21]. Therefore, there are two basic subpopulations of macrophages – classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages, through the secretion of pro-inflammatory cytokines (the most important of which are IL-6, IL-12 and TNF-a) and chemokines and through the presentation of antigens, promote an inflammatory response and exhibit antitumour activity. On the other hand, M2 polarised macrophages, also called tumour-associated macrophages (TAMs), have a very weak antigen-presenting ability and, through the secretion of arginase, IL-10, TNF-b and other cytokines, have a role in reducing inflammation, are significant in tissue repair and wound healing, and contribute to tumour growth[22].

Macrophages have been recognised as an important cell population of the inflammatory infiltrates of the microenvironment of malignant tumours[23]. They are attracted by numerous factors, such as hypoxia, high cell turnover and similar, with the aim of participating in establishing tissue homeostasis[20]. However, this results in a maladaptive response that, instead of suppressing tumour growth and progression, promotes tumour growth by initiating the process of angiogenesis, tissue remodelling and by establishing an immunosuppressive environment[24]. Therefore, there is an increasing number of studies that examine the role of macrophages in carcinogenesis, the prognostic and predictive significance of their presence and abundance in the inflammatory infiltrate of the tumour microenvironment, and also, the possibility of immunotherapeutic intervention on the M2 population of macrophages.

In a study by Chen el al[18], assuming that an HPV infection promotes the polarisation of M2 macrophages, CD613 was used along with CD68, and a positive association was shown between the

expression of both markers and the cervical carcinogenesis. Some scholars[25], who dealt with the protein expression of macrophages polarisation *in situ*, point out that CD163 cannot be used as an independent M2 differentiation marker, and that it would be desirable for it to be used for these purposes alongside some other markers, such as CMAF.

Research also show that macrophages of both phenotypes can be present to different degrees inside the stroma of the same tumour. Therefore, for instance, TAMs with a high expression of major histocompatibility complexes (MHC) class-II molecules can be limited to normoxic zones of a tumour and express M1 markers and anti-angiogenic chemokines, while in rest of the tumour TAMs with a classical M2 phenotype and a low MHCII molecule expression can dominate[26]. As a good example of a tumour with a hybrid phenotype of macrophages, in which expression of pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6, but also CCL2, was shown, renal cell cancer is frequently stated[27].

For all these reasons, further investigation of macrophage polarisation *in situ*, through the simultaneous application of a larger number of markers, both in the tumour stroma and in premalignant lesions, could be interesting.

By reviewing the literature on the subpopulation of T lymphocyte expression in dysplastic lesions and cervical carcinoma, we found that some authors show an increase of the number of CD8 and CD4 lymphocytes with the lesion grade[28]. Other studies show a connection between a large number of CD4 and CD8 lymphocytes[29,30] and lesion regression, while the largest number of studies show downregulation of both the subpopulation of T lymphocytes in premalignant lesions and cervical carcinoma, emphasising the importance of local immunosuppression on the evolution of HPV-induced changes[31,32].

In our study no correlation was found between the level of CD8 lymphocyte expression and the presence of dysplasia, but a positive correlation between CD4 expression and LSIL was shown. Given the well-known observation that a higher percentage of LSIL lesions is a subject of resolution in relation to HSIL[33,34], this finding could explain the higher percentage of CD4 lymphocytes in an inflammatory infiltrate of early dysplastic lesions. Unfortunately, our study was limited by the inability to monitor patients and the potential regression in time. Similarly to macrophages, we did not find a connection between the level of CD4 lymphocyte expression and the presence of HR-HPV infection in samples stratified in relation to the lesion grade.

With the exception of several cases in which a high level of CD20 lymphocyte expression was noted, in our samples, a small number of B cells was registered overall, and no correlation was found, as already mentioned, between their presence with HR-HPV genotypes, or with the presence of dysplastic lesions. The absence of B cells in the inflammatory infiltrate does not exclude the possibility of an antibody effect directed towards molecules expressed on the surface of infected or transformed cells.

Conclusions

Our results show a higher prevalence of CD4 lymphocytes in early dysplastic lesions compared to a normal cervix and advanced dysplastic lesions, as well as a connection between the macrophage expression levels and the degree of dysplastic lesions, both irrespective of HR-HPV status. We consider these findings a response to dysplasia rather than to HR-HPV infection itself. The increase of the macrophage expression levels with the degree of the lesion speaks in favour of their potential role in the progression of the neoplastic process. Further in situ studies of the composition of the inflammatory infiltrate with reference to other lymphocyte subpopulations, macrophage subpopulations and other cell types, such as Langerhans cells, could contribute to a better understanding of the tissue response to HPV infection and dysplasia.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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