Original paper

Differential expression of NLRP3 inflammasome genes in HPV infected cervical cells

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Abstract

The aim of this study was to to evaluate and correlate the expression of some actors of canonical inflammasome pathway with the degree of cervical lesions in cytologic specimens.

Methods: The study group included 128 Romanian female patients aged between 16 and 68 years that were referred to Synevo laboratories for performing both a cytological evaluation and HPV genotyping.

Results: Out of 128 analysed patients 112 (87.5%) had a positive HPV result (with one or more HPV genotypes), as follows: 34 (26.6%) patients with high-risk HPV (hrHPV) genotype(s) only, 36 (28.1%) cases with low-risk HPV (lrHPV) genotype(s) only, and 42 (32.8%) patients with combined hrHPV and lrHPV (hrHPV+lrHPV) genotypes. Our study indicate the possibility that NLRP3 inflammasome is differentially activated in HPV-infected cervical cells according to HPV oncogenic potential (hrHPV versus lrHPV) and degree of cervical lesions.

Conclusion: NLRP3-independent mechanisms, such as decrease of IL1B and IL18 transcription in association with high-grade cytological lesions, could be part of the immune escape strategy used by HPV to ensure its persistence in the host cervical cells.

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Introduction

The innate immune response is very important in the defense against infectious agents and also in the deal with cancer. Dangerous signals are sensed by the immune system leading to the activation of inflammasome. To ensure their persistence, many oncogenic viruses developed different strategies to evade from immune surveillance. Human Papilloma Viruses (HPVs), the etiological factor of cervical cancer, are a redoubtable example. The infection with HPV has some specific features such as genotype-specific host-restriction and genotype-specific tissue-preferences (DOORBAR & al [1]). The virus infects the basal layer of mucosa through lesions and, for the production of progeny, the virus needs the differentiated layers of epithelial tissue in which the viruses are multiplied, assembled and eliminated as squams (DOORBAR & al [1]). In abortive infections, the virus remains in basal cells and multiplies with it leading to the development of neoplasia at specific epithelial sites (EGAWA & al [2]; DOORBAR [3]). The switch from productive to abortive infection is accompanied by the deregulated expression of the high-risk E6 and E7 proteins through a mechanism in which the host's immunity, epithelial environment, cell type play an important role (DOORBAR & al [1]; EGAWA & al [2]). In cervical epithelial cells, the virus coexists as an episomal extra-chromosomal element and as integrated HPV DNA within the host cell, a form that is essential for pro-carcinogenesis, but insufficient to produce progression to transformation (AGIUS [4]). The deregulated immune responses associated with HPV persistent infection can lead to severe injury and consequent carcinogenesis. The HPV is a common infection in women, but cervical cancer rarely arises because most of the infections are cleared by a cell-mediated immune response (DOORBAR & al [1]) and inflammasome activation as an essential link to adaptive immune responses (SO & al [5]).

Cancer cells have developed a lot of strategies to limit cell death, a mechanism that acts as a protector in normal cells (HASSAN & al [6]). Pyroptosis is a regulated cell death form with a pro-inflammatory component, in contrast to apoptosis that is considered immunologically silent. On the other hand, unlike apoptosis, pyroptosis shows a very particular form of DNA damage (FANG & al [7]), characterized by chromatin condensation and DNA fragmentation while the nuclei remain intact (JORGENSEN & al [8]). Also, pyroptosis implies the formation of pores in the plasma membrane, between 1.1 and 2.4 nm in diameter (AACHOUI & al [9]), by a mechanism dependent on the activation of caspase-1 (JORGENSEN & al [8]; BERGSBAKEN & al [10]). Inflammatory caspases are activated by the inflammasome-initiating sensors such as NOD-like receptor protein 1 (NLRP1), NOD-like receptor protein 3 (NLRP3), NLR family CARD domain-containing protein 4 (NLRC4), absent in melanoma 2 (AIM2) or pyrin (MAN & al [11]). In a canonical inflammasome pathway, NLRP3 recruits and binds to inflammasome adapter protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) and activates caspase-1 (FANG & al [7]). Activation of caspase-1 by NLRP3 inflammasome pathway results in the cleavage of gasdermin D (GSDMD) (WANG & al [12]) and proteolytic maturation and extracellular release of its cytokine substrates interleukin-1β (IL-1β) and interleukin-18 (IL-18) (VANDE & al [13]; MAN & al [14]). The N-terminal fragment of cleaved GSDMD (GSDMD-NT) generates pores in the plasma membrane favouring the release of intracellular components (pro-inflammatory cytokines, toxins, endogenous ligands, alarmins, etc.) into the extracellular environment (FANG & al [7]; MAN & al [14]; LIN & al [15]). Pro-inflammatory cytokines IL-1α and IL-1β rapidly increase mRNA expression of hundreds of genes in several different cell types (WEBER & al [16]). Both IL-1β and IL-18 are key mediators of innate and adaptive immune responses with significant effects on tumour growth, invasiveness, and metastasis (WANG & al [12]).

Taking in account that HPV has numerous mechanisms to escape from immune surveillance, our aim was to evaluate and correlate the expression of some actors of canonical inflammasome pathway with the degree of cervical lesions in cytologic specimens.

Materials and Methods

The study group included 128 Romanian female patients aged between 16 and 68 years that were referred to Synevo laboratories for performing both a cytological evaluation and HPV genotyping. After obtaining the informed consent from the patients, cervical samples were collected according to Stroe et al. (2019). Briefly, the sampling of ectocervical, endocervical and transformation-zone cells for cervicovaginal cytology was done using a soft cervical brush (Cervex-Brush®, Rovers Medical Devices B.V., Oss – The Netherlands) and placed into the PreservCyt for adequate transportation of the sample. For RNA and DNA isolation, the samples were concentrated by centrifugation at 2000 rpm and kept at -80°C until further processing.

The liquid-based cytology

Liquid-based technology on TriPath platform (Becton Dickinson, USA) was employed for the preparation and staining of cervical smears. Each sample was prepared in triplicate, evaluated using a digital image processing system (BD FocalPoint™ GS Imaging System, UK) and reported according to the 2001 Bethesda System terminology.

HPV genotyping

DNA extraction was performed automatically on MagNA Pure 96 System (Roche Molecular) using Ampilute Liquid Media Extraction Kit (Roche Molecular). HPV genotyping was performed with Linear Array kit (Roche Molecular) according to manufacturer's indications. This assay detects 37 genotypes including HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 MM8), IS39 and CP6/108. In the

first step DNA targets were amplified by PCR technique using a group of primers specific for the HPV L1 genomic region. For genotyping, each amplified DNA sample was hybridized on a strip containing specific probes in a linear arrangement. The endogenous human beta globin was employed as internal control. The detection limit of the working method is 120, 815, 1920 and 251 copies/mL for genotypes 16, 18, 31 and 45.

Total RNA isolation

Total RNA was isolated using TRIzol™ Reagent kit (Invitrogen, USA) according to manufacturer's recommendations. After homogenization of the sample with 1 ml of TRIzol™ reagent for 5 minutes to allow complete dissociation of the nucleoprotein complex, 0.2 ml of chloroform was added to separate RNA from DNA and proteins. The samples were centrifuged for 15 min at 12000 x g at 4°C and the colourless, upper aqueous phase containing RNA was transferred in a new tube. RNA was extracted using 0.5 ml of isopropanol, washed in 70% ethanol and dried for 5-10 minutes. RNA was resuspended in 20 µl of RNase free water and RNA's concentration was quantified spectrophotometrically using NanoDrop 1000 (Thermo Scientific, USA). The RNA was kept at -80°C until reverse transcription was performed.

Reverse transcription (RT) of total RNA

High-Capacity RNA-to-cDNA™ kit (Applied Biosystems, USA) was used to reverse-transcribe 2 µg of total RNA into cDNA in 20 µl reaction volume. The reverse transcription program included the following steps: step1 – 10 min at 25°C, step 2-120 min at 37°C, step 3-5 min at 95°C. The reaction was stopped by heating at 95°C for 5 minutes followed by maintaining at 4°C. The cDNA ready for use in Real-time PCR was kept at -20°C until the amplification reaction was performed.

Real-time PCR

TaqMan primers and probes and PCR master mix (Applied Biosystem, USA) were employed to evaluate the expression of *NLRP3* (Hs00248187_m1), *CASP1* (caspase-1) (*Hs00354836_m1*), *IL1B* (*Hs01555410_m1*), and *IL18* (*Hs01038788_m1*) genes. PCR amplification was performed following the standard PCR program of StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific, Waltham, USA). For the normalization of Real-time RT-PCR data the double delta Ct ($\Delta\Delta$Ct) method was used. The housekeeping gene GAPDH was taken as reference; the group of patients with normal cytology and undetectable HPV represented the control for relative mRNA quantification of target genes in each patient sample. To obtain the fold change in expression levels the $2^{-\Delta\Delta Ct}$ was calculated. Results were displayed as $\log_2$ fold change.

Statistical analysis

Statistical analysis was performed using version 6.01 of GraphPad Prism software (GraphPad, San Diego, USA). Differences in relative mRNA expression levels among more than two groups of patients were assessed by the non-parametric Kruskal-Wallis test followed by post-hoc Dunn's multiple comparisons test. Mann-Whitney U test was employed for evaluating the differences in transcript levels between two group of patients. *P* values 0<0.05 were considered statistically significant.

Results

Baseline characteristics of patients

Patients were stratified by the type of cervical cytological lesions and HPV status (Table 1). According to Bethesda classification the following cytological results were obtained for the group of 128 patients: 45 (35.2%) NILM (negative for intraepithelial lesion or malignancy), 36 (28.1%) ASC-US (atypical squamous cells of undetermined significance), 40 (31.2%) LSIL (low-grade squamous intraepithelial lesion), and 7 (5.5%) HSIL (high-grade squamous intraepithelial lesion). Out of 128 analysed patients 112 (87.5%) had a positive HPV result (with one or more HPV genotypes), as follows: 34 (26.6%) patients with high-risk HPV (hrHPV) genotype(s) only, 36 (28.1%) cases with low-risk HPV (lrHPV) genotype(s) only, and 42 (32.8%) patients with combined hrHPV and lrHPV (hrHPV+lrHPV) genotypes. A number of 10 patients displayed normal cytology and negative HPV result at genotyping serving as the control group.

Table 1. The distribution of patients according to the type of cervical lesions and HPV detection

HPV status	Cytology			
	NILM n=45 (%)	ASC-US n=36 (%)	LSIL n=40 (%)	HSIL n=7 (%)
HPV (-), n=16	**10 (22.3)**	**6 (16.7)**	**0**	**0**
HPV (+), n=112	**35 (77.7)**	**30 (83.3)**	**40 (100)**	**7 (100)**
hrHPV, n=34	8 (17.8)	10 (27.8)	12 (30.0)	4 (57.1)
hrHPV+ lrHPV, n=42	11 (24.4)	11 (30.5)	17 (42.5)	3 (42.9)
lrHPV, n=36	16 (35.5)	9 (25.0)	11 (27.5)	0

NLRP3 expression

In the case of *NLRP3* mRNA expression cytological samples positive for HPV displayed a median $\log_2$ fold change of 4.98 relative to controls. Gene expression levels were significantly different among various cytological groups (*P*=0.0004; Kruskal–Wallis *H* test), median $\log_2$ fold change values being higher in ASC-US and LSIL compared to NILM HPV (+) cases (6.61 versus 1.74;

6.11 versus 1.74, respectively, Dunn's multiple comparisons test, Figure 1A). Normalized *NLRP3* mRNA levels differed also between HPV groups ($P=0.0031$; Kruskal–Wallis *H* test); the highest median $\log_2$ fold change value was observed in hrHPV, followed by combined hrHPV and lrHPV group, the differences being statistically significant when compared to the median $\log_2$ fold change of lrHPV group (6.21 versus 2.07; 5.14 versus 2.07, respectively, Dunn's multiple comparisons test, Figure 1B). Although the subgroup of LSIL positive for hrHPV seemed to present higher *NLRP3* expression levels than HSIL the difference did not present statistical significance (6.99 versus 4.28, $P=0.1203$, Mann-Whitney *U* test, Figure 1C).
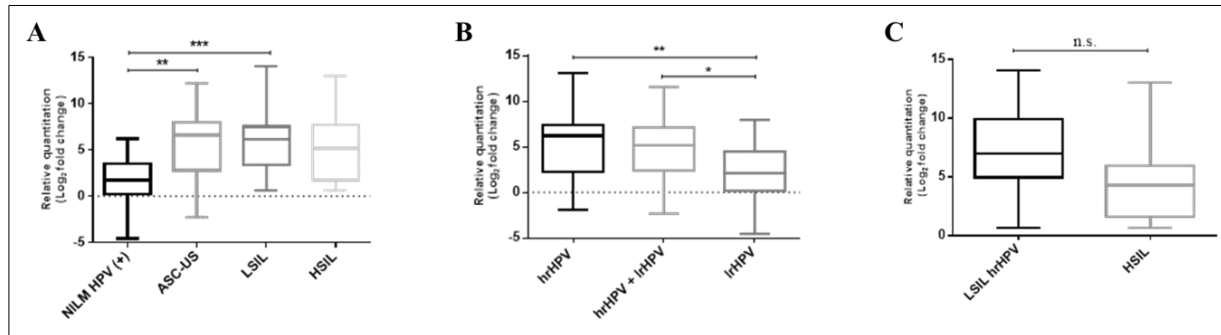

Figure 1. *NLRP3* mRNA normalized expression levels in patients with various cytological results **(A)**, HPV groups **(B)**, hrHPV-associated LSIL and HSIL **(C)**. Each box plot displays the median, interquartile range, minimum and maximum $\log_2$ fold change values, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s. not significant.

CASP1 expression

In the whole batch of cervical specimens positive for HPV a median $\log_2$ fold change of 1.39 versus control group was observed when analysing *CASP1* mRNA expression levels. No statistically significant differences were registered among cytological or HPV groups ($P=0.5917$, $P=0.3005$, respectively; Kruskal–Wallis *H* test, Figures 2A and 2B). The comparison between LSIL positive for hrHPV and HSIL showed a tendency towards a higher *CASP1* relative expression levels in the LSIL subgroup, without reaching statistical significance (median $\log_2$ fold change of 3.33 versus 0.42, $P=0.08$, Mann-Whitney *U* test, Figure 2C).
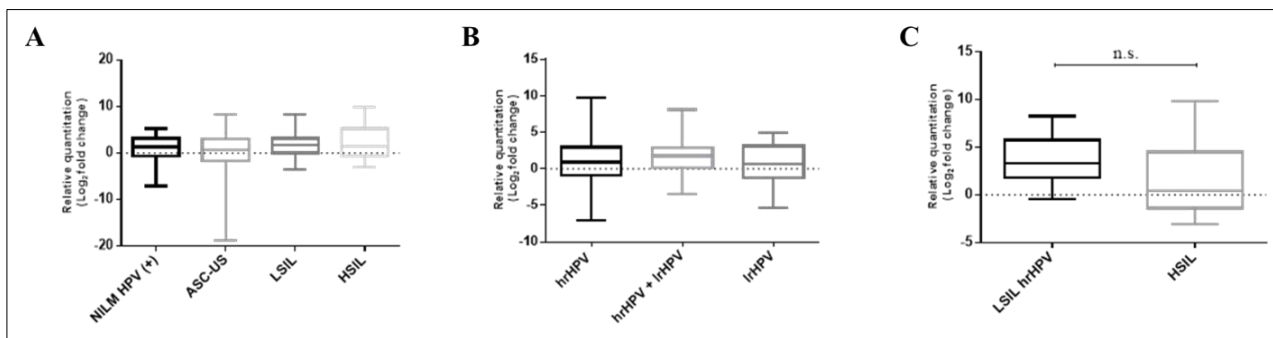

Figure 2. *CASP1* mRNA normalized expression levels in patients with various cytological results **(A)**, HPV groups **(B)**, hrHPV-associated LSIL and HSIL **(C)**. Each box plot displays the median, interquartile range, minimum and maximum $\log_2$ fold change values, n.s. not significant.

IL1B expression

In the next step we analysed the transcript levels of *IL1B* and we found a median $\log_2$ fold change value of 1.1 in HPV positive samples relative to controls. No significant differences in *IL1B* gene expression among cytological or HPV groups ($P=0.098$, $P=0.308$, respectively; Kruskal–Wallis *H* test, Figures 3A and 3B) were present. However, when LSIL positive for hrHPV and HSIL groups were considered separately the median $\log_2$ fold change of *IL1B* expression level was significantly lower in HSIL (-0.94 versus 2.67, $P=0.043$, Mann-Whitney *U* test, Figure 3C).
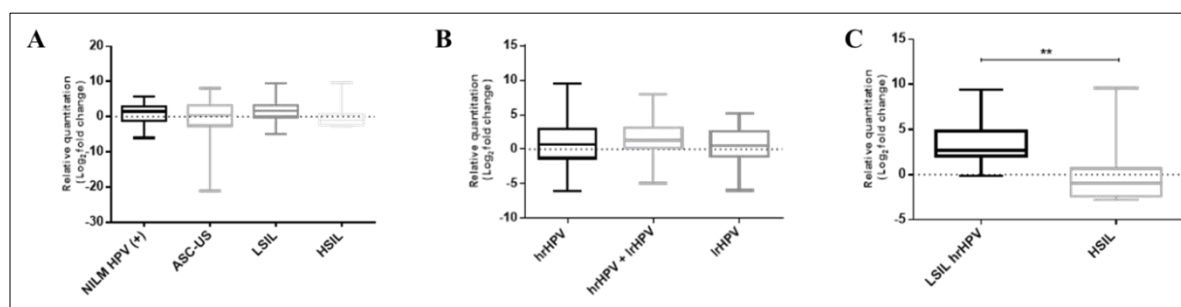
Figure 3. *IL1B* mRNA normalized expression levels in patients with various cytological results **(A)**, HPV groups **(B)**, hrHPV-associated LSIL and HSIL **(C)**. Each box plot displays the median, interquartile range, minimum and maximum log₂ fold change values, **P < 0.01.

IL18 expression

The expression levels of *IL18* gene were also assessed and HPV positive samples exhibited a median log₂ fold change value of 1.71 compared to control group. Neither cytological nor HPV groups presented significant differences in respect to *IL18* transcript levels (*P*=0.1029,

P=0.4428, respectively; Kruskal–Wallis *H* test, Figures 4A and 4B). Similarly with *IL1B*, the median log₂ fold change of *IL18* expression level was significantly reduced in HSIL in comparison with LSIL positive for hrHPV (-2.87 versus 3.86, *P*=0.043, Mann-Whitney *U* test, Figure 4C).
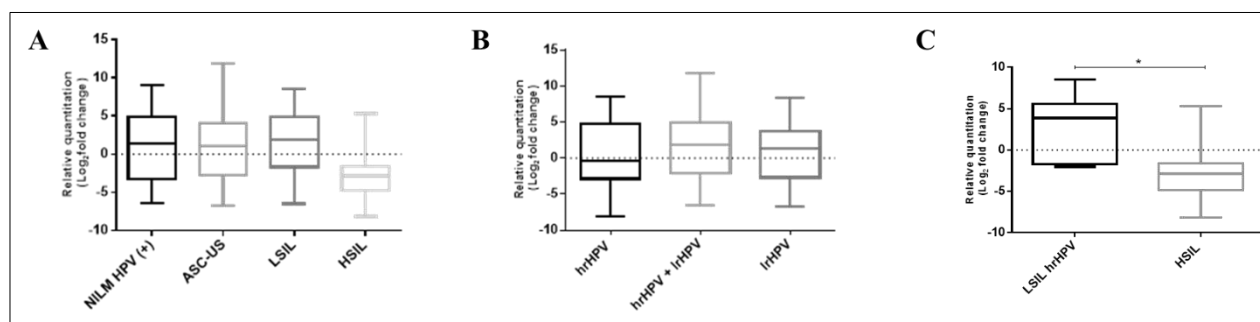

Figure 4. *IL18* mRNA normalized expression levels in patients with various cytological results **(A)**, HPV groups **(B)**, hrHPV-associated LSIL and HSIL **(C)**. Each box plot displays the median, interquartile range, minimum and maximum log₂ fold change values, *P < 0.05.

Discussions and Conclusions

Our data indicate the possibility that NLRP3 inflammasome is differentially activated in HPV-infected cervical cells according to HPV oncogenic potential (hrHPV versus lrHPV) and degree of cervical lesions. Concurrently, it is suggested that development of high-grade lesions is associated with a reduction of *IL1B* and *IL18* gene expression levels, providing insights into the mechanisms of hrHPV immune evasion and persistence.

The involvement of NLRP3 pathway in HPV-host interaction was previously suspected due to identification of *NLRP3* rs10754558 polymorphism that was linked with a lower risk of hrHPV infection as well as with protection against virus persistence (PONTILLO & al [17]).

As a first observation, the expression of *NLRP3* mRNA was significantly higher in patients with hrHPV infections compared to lrHPV and combined infections,

and also in cases of low-grade cytology (ASC-US and LSIL) versus NILM with HPV positivity. Due to the fact that ASC-US and LSIL are generally associated with productive HPV infections (EBISCH & al [18]) that in 90% of cases are eliminated by the host immune system within two years (TAI & al [19]), our finding might reflect the high potential of HPV clearance in women with low-grade cytology.

Interestingly, *CASP1*, *IL1B* and *IL18* genes have a different pattern of expression among the analysed groups compared to *NLRP3*, suggesting that NLRP3-independent mechanisms influence also the expression of these inflammasome components during HPV infection phases. In line with this observation, in a previous study that aimed to characterize the innate responses triggered by HPV in infected keratinocytes it was found that HPV DNA induced activation of AIM2, a cytosolic sensor of pathogen DNA, leading to inflammasome formation and subsequent release of IL-1β and IL-18 (REINHOLZ & al [20]).

We found a significantly reduction of *IL1B* and *IL18* mRNA levels in HSIL compared to hrHPV induced LSIL. It is largely known that in terms of outcome there are essential differences between LSIL and HSIL. While up to 60-80% of all LSIL cases register a spontaneous regression within 2-5 years and a low rate of progression to biopsy-confirmed high-grade lesions (CIAVATTINI & al [21]), HSIL is associated with abortive hrHPV infections and poses a higher risk of progression to invasive disease (SENAPATI & al [22]). On the other hand, evolving from LSIL to HSIL is associated with hrHPV integration into the host DNA and overexpression of E6 and E7 oncoproteins (SENAPATI & al [22]). It seems that E6 oncoprotein is involved in the viral immune escape by blocking *IL1B* transcription through the inhibition of Interferon Regulatory Factor 6 (IRF6), essential for *IL1B* promoter activation (AINOUZE & al [23]). Although HPV16 – HPV genotype detected with the highest frequency in cervical premalignant and malignant lesions – was able to induce the inflammasome activation and pyroptosis in human keratinocytes in early stages of infection, upon induction of E6 oncoprotein *IL1B* expression was suppressed, without affecting *NLRP3*, *AIM2* and *CASP1* transcript levels. The same effect of *IL1B* transcription inhibition was observed in neoplastic cervical cells (AINOUZE & al [23]). In agreement with our data showing a significant downregulation of *IL18* mRNA levels in HSIL compared to LSIL, a previous study indicated that *IL18* transcript levels, assessed by Northern blot and RT-PCR, were decreased in HPV-negative C-33A cervical cancer cell line after transfection with E6 oncogene (CHO & al [24]). Further supporting our data, Matamoros et al. showed that the risk of progression of premalignant lesions to cervical cancer was more than double in patients with reduced *IL1B* and *IL18* gene expression levels (MATAMOROS & al [25]). The involvement of inflammasome components IL-1β and IL-18 in HPV infection and cervical lesion progression was also confirmed at genetic level, *IL1B* rs1143643 and *IL18* rs1834481 polymorphisms being related to protection against HPV infection and/or persistence (EBISCH & al [18]).

Our study has several limitations. Firstly, due to low amount of cytological specimens we were not able to perform protein extraction and Western blot analysis in order to confirm our RT-PCR results. Secondly, no bacteriological studies were available for our cases, thus we are aware that, to some extent, coinfections with bacterial pathogens might have exerted in influence on NLRP3-inflammasome component expression.

In summary, we showed that a variable degree of NLRP3 inflammasome activation might occur during different phases of HPV infection and cervical premalignant lesion development. However, other NLRP3-independent mechanisms, such as decrease of *IL1B* and *IL18* transcription in association with high-grade cytological lesions, could be part of the immune escape strategy used by HPV to ensure its persistence in the host cervical cells.

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