MMP-9/RECK imbalance: a mechanism associated with high-grade cervical lesions and genital infection by Human Papillomavirus (HPV) and *Chlamydia trachomatis*

Running title: MMP-9/RECK imbalance in cervical carcinogenesis

Key words: Matrix Metalloproteinases; RECK protein; Cervical Intraepithelial Neoplasia; Human papillomavirus 16; *Chlamydia trachomatis*

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Abstract

Background: Matrix metalloproteinases (MMPs) are important enzymes in the tumor microenvironment associated with progression of cervical intraepithelial neoplasia (CIN) towards squamous cell carcinoma (SCC) of the cervix. However, the role of MMPs in the inflammatory process associated with *Chlamydia trachomatis* infection concomitant with the carcinogenic process driven by HPV has not yet been addressed. In the present study we analyzed the estate of the MMP-9/RECK axis in cervical carcinogenesis. **Methods:** The levels of MMP-9 and RECK expression were analyzed by immunocytochemistry in liquid-based cytology samples from 136 women with high-grade cervical lesions (CIN2/CIN3) and cervical SCC diagnosed by LLETZ, and in 196 women without cervical neoplasia or CIN1. Real-Time qPCR was performed to analyze expression of MMP-9 and RECK in fifteen cervical samples. The presence of HPV-DNA and other genital pathogens was evaluated by PCR. **Results:** We found a higher expression of MMP-9 (OR=4.2; 95%CI: 2.2-7.8) and lower expression of RECK (OR=0.4; 95%CI: 0.2-0.7) in women with CIN2/CIN3/SCC when compared to women from the control group (no neoplasia/CIN1). It was also found a statistically significant association between MMP-9/RECK imbalance and infection by alpha-9 HPV and *C. trachomatis*. The prevalence of *Chlamydia trachomatis* infection was significantly higher in women with high-grade cervical disease **(**OR= 3.7; 95%CI: 1.3-11.3). **Conclusions:** MMP-9/RECK imbalance in cervical smears is significantly associated with high-grade cervical diseases and infection by alpha-9 HPV and *Chlamydia trachomatis*. **Impact:** MMP-9/RECK imbalance during cervical inflammation induced by *Chlamydia trachomatis* might play a role in HPV-mediated cervical carcinogenesis.

Introduction

Cervical cancer is etiologically associated with infection with high-oncogenic risk human papillomavirus (HPV) (1). This tumor has the capacity to metastasize to distant tissues and organs, a fact strongly associated with failure of cervical cancer treatment. The evolution of metastasis is a multistep process and its initial stages can be identified in the pre-neoplasic phase (2). Transformation of a noninvasive cervical neoplasia into an invasive cervical carcinoma requires the migration of epithelial cells through the subjacent extracellular matrix, a process in which the matrix metalloproteinases (MMPs) play a critical role by degrading extracellular matrix components (3,4).

MMPs are zinc-dependent endopeptidases involved in various physiological and pathological processes including tissue remodeling, development and regulation of inflammation (5,6). During carcinogenesis, these enzymes promote changes in the stroma and microenvironment contributing to tumor progression (5-7). Furthermore, MMPs are also dependent on local equilibrium with its physiological inhibitors, such as the RECK protein (Reversion-inducing Cysteine-rich protein with Kazal motifs), which regulates MMP-2 and MMP-9 activities (4,7).

Overexpression of MMP-2 and MMP-9 has been shown to occur in invasive cervical carcinoma and high-grade cervical lesions(3,8,9).We have previously shown that HPV16-positive cell lines express higher levels of MMP-2, MT1-MMP and TIMP-

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2 than HPV-negative cell lines. MMP-9 was expressed at very low levels in both HPV-negative and HPV-positive cell lines (10). In order to analyze MMPs status in a system that reproduces *in vivo* conditions, we have previously developed organotypic epithelial cultures with keratinocytes expressing HPV 16 oncoproteins. We have demonstrated that HPV 16 E7 induces pro-MMP-9 activity, and that E6 and E7 coexpression down-regulates RECK protein levels (11). This latter study also demonstrated that, even in small number of clinical samples, there is an inverse correlation between RECK expression and degree of cervical lesion. Furthermore, previous work from our group demonstrated the association between high-grade cervical lesions and high expression of MMP-9 in conventional Pap-smears, especially when the patient had concomitant genital infection (12).

Clinical studies have shown an association between high-grade cervical intraepithelial neoplasia and *Chlamydia trachomatis* infection (13-16). Considering that concomitant genital infections have been assumed to be risk factors for HPV persistence (14), we hypothesized that infection-induced cervical inflammation were capable of influencing the carcinogenic process driven by HPV. One can consider the role of MMPs in inflammatory process, since the involvement of these enzymes has already been demonstrated in organotypic cultures of fallopian tube infected *in vitro* by *C. trachomatis* (17) and in the pathology of ocular trachoma (18). However, within the context of cervical carcinogenesis, this possible relationship has not yet been elucidated. It is biological plausible that chronic infection by *C. trachomatis* causes an unresolved cervical inflammation that may lead to tissue damage, involving an interplay between HPV, MMPs and their inhibitors. Therefore, in the present study we evaluated the role of MMP-9 and RECK expression in cervical lesions and genital infections.

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Materials and Methods

Subject's inclusion and sample collection

This cross-sectional study included 332 women attending the CAISM Women's Hospital (*Centro de Atenção Integral à Saúde da Mulher*), Family Planning of Human Reproduction Unit, in the School of Medical Sciences, State University of Campinas (UNICAMP), from August 2013 to August 2014. The case group was composed of 136 women that underwent large loop excision of the transformation zone (LLETZ) due to high grade cytological abnormalities in referral Pap-smears. This group had a confirmed histological diagnosis of Cervical Intraepithelial Neoplasia grade 2 (CIN2; n=31), Cervical Intraepithelial Neoplasia grade 3 (CIN3; n=80) or Squamous Cell Carcinoma (SCC; n=25). The control group was composed of 196 women who presented at least two previous normal Pap-smears (n=162), cervicitis (n=9) or Cervical Intraepithelial Neoplasia grade 1 (CIN1; n=25) diagnosed by LLETZ. No cases of adenocarcinoma were included. CIN1 cases were included in the control group since low-grade cervical lesions have more similar characteristics to those of a HPV productive benign infection than of a transforming infection (19). Morphological diagnosis of biopsies obtained by LLETZ were assessed according to the International Histological Classification of Tumours (World Health Organization) (20).

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This study was approved by the Ethics in Research Committee of the School of Pharmaceutical Sciences of the University of São Paulo (USP) (ref. 411.692/2013). After signing the informed consent form, women were submitted to a specular examination for the collection of cervical samples, which were preserved partly in Surepath® vials (BD Surepath™, Pap test) for liquid-based cytology (LBC), RNA and DNA extraction, and partly in sterile saline medium for cervical infections diagnosis. We also collected a cervical smear to perform a GRAM-stained bacterioscopy.

An eight-milliliter fraction of the cervical sample collected in the Surepath® vial was centrifuged twice with the addition of 4 mL of gradient wash buffer. The resulting cellular pellet was suspended in 2 mL of Surepath® medium. From this cellular suspension, 200 µL were used in order to prepare the LBC slides, 200 µL were used for DNA extraction (for HPV genotyping) and 200 µL for RNA extraction (for analysis of gene expression).

Immunocytochemistry for MMP-9 and RECK evaluation in LBC slides

Liquid based cytology (LBC) slides were used to determine the expression of MMP-9 and RECK by immunocytochemistry analysis. In order to prepare the LBC slides, we used the automated PrepStain® system (TriPath Imaging, Inc., Burlington, NC). As a positive control of MMP-9 labelling, we used the HT1080 cell line derived from human fibrossarcoma (American Type Culture Collection, Manassas, VA, USA), which was chosen because it is known to be abundant in MMP-9. As a positive control for RECK labelling, we used primary human fibroblasts extracted from human foreskin at the Clinical Cytopathology Laboratory of the School of Pharmaceutical Sciences - USP.

To evaluate the expression of MMP-9, the primary antibody anti-MMP-9 (MAB 13415, Chemicon-Millipore; Temecula, CA, USA) was used at a 1:150 dilution in phosphate buffer saline solution (PBS). To evaluate RECK expression we used the primary antibody anti-RECK (BD 611512, Biosciences, San Jose, CA, USA) also at the 1:150 dilution in PBS. Incubation with primary antibodies was performed overnight in a wet chamber at 4°C. Incubation with secondary antibody and staining with DAB chromogen was performed using the Envision + Systems/HRPP commercial kit (K4007/DAKO Cytomation, CA, USA). After the immunocytochemical reaction, we proceeded to nuclei staining with hematoxylin. Immunocytochemical analysis was performed on 321 LBC slides for MMP-9 protein and on 327 LBC slides for RECK protein.

Criteria for analyzing immunocytochemistry for MMP-9 and RECK in LBC slides

In order to analyze the immunocytochemistry slides, we adapted the criteria described by Li et al. (9). We performed a blind test in which the observer was not informed of the diagnosis of any cervical lesions. The slides were analyzed at 400x magnification using a light microscope. Cells were counted and classified as "positive" or "negative" according to DAB chromogen staining: brown stained cells were considered as "positive" regardless of the intensity of the color (pale-yellow, brown or yellow-brown), and blue stained cells (hematoxylin only) were considered "negative". Counting was performed in two distant random fields, avoiding the specimen edges. In

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case of discrepant scores between these two fields, a third field was counted. Slides

grading was performed as follows: score 1 - 0 to 25% of DAB stained squamous cells;

score 2 - 26 to 50% of DAB stained squamous cells; score 3 - 51 to 75% of DAB

stained squamous cells; score 4 - more than 75% of DAB stained squamous cells. Both

atypical and typical squamous cells were considered in the counting.

At first, the analysis was performed by two independent observers (PCP and

MGD) and, in case of disagreement, a third observer (MZ) was consulted. Scores 1 and

2 were considered as negative or mild expression, and scores 3 and 4 were considered as

moderate or accentuated expression.

***Real-time quantitative PCR (RT-qPCR) technique for evaluation of MMP-9 and
RECK expression in cervical samples***

Total RNA was extracted from cervical samples of four women from the control

group (no neoplasia/CIN1) and from eleven women in the case group (CIN2/ CIN3/

SCC) using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were

quantified by UV light spectrophotometer at 260 nm (ND-1000–NanoDrop-

Wilmington, DE, USA) and RNA quality was verified by the A_{260}/A_{280} ratio. cDNA was

synthesized from 1µg RNA using the Reverse Transcription c-DNA High-capacity® kit

(4388813, AB- Applied Biosystems, Warrington, UK). RT-qPCR was carried out using

Taqman® specific primers for the genes of interest (MMP-9: Hs00234579-m1, RECK:

Hs01019179-m1, Applied Biosystems, Warrington, UK). The assay was performed

using ABI Prism® 7500 Sequence Detection System equipment (Applied Biosystems,

New Jersey, USA), under the following conditions: activation of the AmpErase®UNG

enzyme at 50°C for 2 minutes, followed by initial denaturation at 95°C for 10 minutes,

40 cycles of 15 seconds at 95°C to end the denaturation process, and finally, annealing

and extension of the primers and probe at 60°C for 1 minute. The level of gene

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expression was calculated in relation to the endogenous controls expression (β-actin: Hs01060665-g1 and GAPDH: Hs02758991-g1, Applied Biosystems, New Jersey, USA) using the cycle threshold (Ct) method.

HPV Genotyping

DNA was extracted from 320 cervicovaginal samples collected in Surepath® vial. The cellular suspension was centrifuged and resuspended in 200μL of digestion solution (50mM Tris-HCL, 1mM EDTA, and 0.5% Triton X-100) + 200 μL TEP buffer (200μg/mL of proteinase K in TE). After 24 hours of digestion at 55^0C, the nucleic acids were purified with Phenol:Chloroform:Isoamyl Alcohol (25:24:1) followed by precipitation with 3M sodium acetate plus 2.5 volumes of absolute ethanol, and washing with 70% ethanol. The DNA pellet was re-suspended in 50μL of water.

For HPV genotyping, we used the Linear Array Genotyping HPV kit (Roche Molecular Systems Inc., Pleasanton, CA, USA) according to the manufacturer's protocol. This test uses target-DNA amplification by PCR and nucleic acids hybridization, detecting 37 types of low and high risk HPVs: HPV 6, 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, 81, 82, 83, 84, IS39, and CP6108. Twelve women were excluded from HPV genotyping because of inadequate internal controls (β-globin).

Multiplex PCR for seven genital infections

Multiplex PCR (M-PCR) was performed for the diagnosis of seven concomitant genital infections in cervical samples in accordance with a previously standardized and validated method (21). The diagnosed concomitant genital infections were: *Chlamydia*

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trachomatis, Herpes virus simplex 1 and 2 (HSV1 and HSV2)*, Neisseria gonorrhoeae, Mycoplasma hominis, Trichomonas vaginalis* and *Treponema pallidum*. This analysis was performed at the Clinical Cytology Laboratory of the State University of Maringá (*Laboratório de Citologia Clínica da Universidade Estadual de Maringá,* UEM, Paraná, Brazil), using endocervical samples collected in sterile saline solution. DNA extraction of these samples was performed using the PureLink® RNA-DNA viral purification mini kit (12280-150, Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. Briefly, primers were characterized by compatible melting temperatures and yielded amplicons with sizes easily separable by polyacrilamyde gel electrophoresis. The protocol consisted of a reaction mixture of 25 µL containing 2.5 mM of each dNTP, 0.6 mM of $MgCl_2$, 25 mM of the specific primer, 5 µL of extracted DNA (50 ng of total sample) and 1 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The PCR conditions comprised of 35 amplification cycles of denaturation for 10 minutes at 94°C, annealing for 1 minute at 62°C, extension for 1 minute at 72°C and final extension for 10 minutes at 72°C (Thermal cycler, Biosystem, CA, USA). The M-PCR products were analyzed in 8% polyacrylamide gels (**Figure 1**). Positive controls for all genital infections were derived from positive clinical samples diagnosed by reference methods, including culture and/or single PCR (sPCR) (22). Human β-globin-specific primers GH20/PC04 were used as internal controls for amplification and DNA integrity. The diagnosis of infections by multiplex PCR was not determined in 47 samples because of inadequate DNA internal control (β -globin).

GRAM stained bacterioscopy

The GRAM-stained slides were analyzed using the criteria described by Nugent et al. (23), which applies a scoring system for the presence of bacterial morphotypes of *Gardnerella vaginalis*, *Lactobacillus sp* and *Mobiluncus sp*. A score of 0-3 consisted of

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normal microbiota, a score of 4-6 consisted of moderately disturbed microbiota, and a score equal or greater than 7 consisted of bacterial vaginosis (BV).

Statistical analysis

The association between cervical lesions, genital infections, MMP-9 and RECK expression was evaluated by odds ratio (OR) with a 95% confidence interval (CI). The social and demographic characteristics were evaluated by Chi-square test. Multiple logistic regression analysis was used to calculate the odds ratios adjusted for the control variables, such as age, age at first sexual intercourse, ethnicity, parity, smoking and genital infections. The statistical analyzes described above were performed using the software SAS version 9.1.3 (SAS Institute, Cary, NC, EUA). The RT-qPCR gene expression of MMP-9 and RECK data was evaluated by Student's T test, using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, EUA). Significance level was defined at 5% for all statistical analysis.

Results

In the present study, the mean age of women was 35.4 ± 10 years in the case group (CIN2/CIN3/SCC) and 35.8 ± 8 years in the control group (no neoplasia/CIN1), with no statistical difference between the two groups. The profile of social and demographic characteristics in the study groups is shown in **Table 1**. We found a statistically significant higher frequency of smoking women in the case group when compared to the women in the control group (37% vs. 21%, respectively; p=0.001). We also found a higher frequency of women having had first sexual intercourse at age below 15 years in the study group (44%) when compared to the women in the control group (30%), and this difference was statistically significant (p=0.008).

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We were able to gather data on a large spectrum of diagnosed genital infections from 300 women, by GRAM and M-PCR techniques. The most prevalent genital infection was BV (23%), followed by *C. trachomatis* (11%), *Trichomonas vaginalis* (3%) and *Candida* spp (3%). *Neisseria gonorrhoeae* was detected in four women (1.3%), while HSV1, HSV2 and *Treponema pallidum* were identified in two women each (0.9%). One case of *Treponema pallidum* was associated with an infection by *C. trachomatis.*

The overall frequency of HPV was 52% (166/320). Of the 166 positive cases, 32% presented multiple HPV infections and 68% presented a single infection. We detected 32 different types of HPV. The most prevalent type detected was HPV16 (34%), followed by HPV31 (8%), HPV52 (7%), HPV33 (6.5%), HPV58 (6%), HPV35 (5.5%), and HPV62 (4%). HPV18 represented 2.6% of the cases. In the case group (CIN2/CIN3/SCC), the frequency of HPV was 89%, while in the control group (no neoplasia/CIN1) the frequency was 24%. Regarding the oncogenesis risk of HPV, 91% of the HPV-positive cases were high-oncogenic risk types, 7% were low-oncogenic risk types and 2% were probably oncogenic.

With regard to the phylogenetic distribution of HPV, infection by alpha-9 species (which includes HPV types 16, 31, 33, 35, 52, 58) occurred in 75% of the cases, and infection by alpha-7 species (HPVs 18, 39, 45, 56, 59, 66, 68) occurred in 9% of the cases, while a combination of these two species was found in 5% of all cases. Other HPV species were inexpressively detected such as alpha-5, alpha-6, alpha-11 and alpha-15.

The relationship of the HPV status with the MMP-9 and RECK expression is shown in **Table 2**, while the relationship between HPV and other infectious agents is represented in **Table 3.** We demonstrated that the presence of HPV, especially HPV16

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and other alpha-9 HPV types, was significantly associated with increased expression of MMP-9 and low expression of RECK (**Table 2**). Women infected by *C. trachomatis* were more likely to have a moderate/accentuated MMP-9 expression, which was about 2.6 times higher than the cases negative for *C. trachomatis*, with statistically significant results even after adjusting for the presence of HPV (OR= 2.6; 95%CI: 1.1-6.7). Furthermore, women with *C. trachomatis* had a higher prevalence of HPV multiple infection (OR= 2.7; 95%CI: 0.8-9.0). We also found a statistically significant association between *C. trachomatis* and high-grade cervical disease (CIN2/CIN3/SCC) when compared to the control group, i.e. no neoplasia/CIN1 cases (OR=3.7; 95%CI: 1.1-4.9). However, this association was not seen for BV or other genital infections in general (**Table 3)**

When we considered only women infected by high-risk HPV, we found that frequency of moderate/accentuated MMP-9 expression in women with concomitant infection by *C. trachomatis* was higher (17%) when compared to negative-mild MMP-9 expression (6%), although this result was not statistically significant (OR=3.3; CI 95%: 0.7-15.8). Moreover, frequency of moderate/accentuated RECK expression in *C. trachomatis* positive samples is lower (7%) when compared to negative-mild RECK expression (16%), but this difference was also not significant (OR= 0.4; CI 95%: 0.7-1.7) (data not shown).

One important finding reported in **Table 4** is that women from the case group (CIN2/CIN3/SCC) had a greater frequency of moderate/accentuated expression of MMP-9 than the women in the control group (no neoplasia/CIN1) and this association was statistically significant (OR= 4.2; 95%CI: 2.2-7.8). Additionally, when evaluating the expression of MMP-9 for each type of neoplasic cervical lesion in comparison with women without cervical neoplasia, we observed an increase in risk of

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moderate/accentuated expression in higher grades of CIN. **Table 4** also shows that there is a strong association between CIN3 and moderate/accentuated expression of MMP-9 (OR=5.4, 95% CI 2.4-12.4). When considering RECK expression this relation is inverted: lower RECK expression is associated with higher grades of CIN. These results were also statistically significant after a multivariate analysis adjusted for the study variables, such as age, age at first sexual intercourse, ethnicity, parity, smoking and genital infections.

Figure 2 represents the immunocytochemical staining for MMP-9 and RECK proteins, showing that there is a clear increase in the intensity and number of cells stained for MMP-9 in CIN3 lesions (**Figure 2B**) when compared to normal cervical smears (**Figure 2A**). Moreover, we observed a decrease in the expression of RECK in CIN3 lesions **(Figure 2E)** when compared to normal cervical smears **(Figure 2D).**

Figures 2C and 2F show a graphic representation of the MMP-9 and RECK mRNA expression evaluated by RT-qPCR. We observed that, women in the case group (CIN2/CIN3/SCC) presented a higher MMP-9 mRNA expression than women in the control group (no neoplasia/CIN1), but this result was not statistically significant (p=0.2). On the other hand, there was a statistically significant lower RECK mRNA expression in the case group when compared to the control group (p=0.03). These results are consistent with the ones we found in the immunocytochemical analyzes.

Discussion

In this study, we presented MMP-9 and RECK expression analysis in LBC cervical samples of women with cervical intraepithelial neoplasia and cervical squamous cell carcinoma, concomitant with other genital infections. We found that higher expression of MMP-9 and lower expression of RECK is strongly associated with high-grade cervical lesions and with the presence of alpha-9 HPVs, which includes

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HPV 16. Additionally, we demonstrated, for the first time to the best of our knowledge, that *C. trachomatis* infection is associated with MMP-9/RECK imbalance. This may constitute a possible mechanism by which inflammation contributes to HPV-mediated carcinogenesis. Furthermore, our results are in accordance with data from the literature that has already described the relation between *C. trachomatis* infection and high-grade cervical lesions (13-16).

In a previous study from our group, da Silva Cardeal et al. (10) discussed that there is no correlation between RECK expression and low MMP activity in HPV16-positive (SiHa, CAsKi) and HPV16-negative (C33A) human cervical carcinoma cell lines. In that model, the authors hypothesized that TIMP-2, but not RECK, is involved in MMP inhibition. However, in the present study, data from clinical samples evidenced an imbalance between MMP and RECK which has already been shown in other human cancers (24-26) and in organotypic epithelial cultures with HPV-infected keratinocytes (11).

Our clinical data is in agreement with the study from Wang et al. (27), which demonstrated that higher expression of RECK and lower expression of MMP-9 and MMP-2 constitutes a positive prognostic survival factor in patients with invasive cervical carcinoma. Gosh et al. (28) investigated the gelatinolytic activity of matrix MMP-2 and MMP-9 in preinvasive and invasive carcinoma of the uterine cervix and concluded that both MMP-2 and MMP-9 have a role in cancer progression and remodeling of the ectocervix. Furthermore, a study conducted in cervical biopsies showed a decrease in RECK expression in preneoplasic and neoplasic lesions (11).

Results from the present study show that women infected by alpha-9 HPVs, which includes HPV16, are more likely to have higher expression of MMP-9 and lower

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expression of RECK when compared to women infected by alpha-7 HPV or other HPV species. In this context, Cardeal et al. (11) indicated that HPV16 E7 expression is associated with increased pro-MMP-9 activity in the epithelial component of organotypic cultures, while E6 and E7 HPV16 oncoproteins co-expression down-regulates RECK and TIMP-2 levels in organotypic cultures. Moreover, clinical studies have demonstrated that women infected by alpha-9 HPV have higher risk of CIN3 development or CIN2 clinical progression, when compared to women infected by alpha-7 HPV (29,30).

Altogether, we can conclude that the analysis of MMP-9 expression in women infected by HPV could be an important tool to be used in clinical practice. Herein, we simultaneously investigated MMP-9, RECK and HPV in liquid-based cytology samples, which is a simple screening method that allows morphological and molecular analyzes in the same sample. Not all women infected by HPV will present a clinically important lesion or will develop cervical carcinoma (19), therefore it is necessary to search for new biomarkers that could positively impact the efficacy and screening outcome of the Pap-test.

It is also important to consider other factors that might interfere in the regulation of such biomarkers; therefore, in this study, we analyzed the effect of concomitant genital infections on the protein expression of MMP-9 and RECK. The presence of genital infection is increasingly considered as a risk factor for the persistence of HPV infection and progression of the associated lesions, and the mechanisms of these associations have been the object of various studies (31-34). In conventional Pap-smears, Matheus et al. (12) showed a high expression of MMP-9 in the presence of genital infections. Herein, we found a significant association between *C. trachomatis* and high-grade cervical lesions (CIN2/CIN3/SCC) (OR: 3.7; 95%:1.3-11.3), which has

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already been amply demonstrated in the literature (13-16). Additionally, we found that women with *C. trachomatis* have a 2.7 higher chance of presenting multiple infections than a single infection by HPV, although this result was not statistically significant in this sample size (OR=2.7, 95% CI: 0.8-9.0). Similarly, Paba et al. (32) demonstrated that the risk of a woman presenting an infection due to two concomitant types of HPV is 7.2 times higher in the presence of *C. trachomati*s. One large longitudinal study also showed that prior infection by *C. trachomatis* is the greatest risk factor for the persistence of HPV DNA (33). Furthermore, Jensel et al. (16) concluded that repeated infections by *C. trachomatis* increase the risk of CIN3 development among women with prevalent and persistent high-risk HPV infection.

The possible causal factors of the association between *C. trachomati*s, HPV and cervical neoplasia are poorly understood. The inflammatory response, cellular hypertrophy and squamous metaplasia caused by *C. trachomatis* infection are thought as possible mechanisms of the association between *C. trachomatis* and cervical neoplasia, since these events promote a cellular turnover that provides an advantage for the carcinogenic process induced by HPV (34). Thus, since MMPs are important mediators of tissue remodeling and inflammation, it is to be expected that they act in the inflammatory process caused by *C. trachomatis.* We found that women infected by *C. trachomatis* had higher MMP-9 expression in relation to women negative for *C. trachomatis*. An earlier study on ocular trachoma induced by *C. trachomatis* showed an increase in inflammatory factors and MMPs, including MMP-9 (18). More recently, another study demonstrated an increase in production of MMP-9 and MMP-2 in organotypic cultures of the fallopian tube infected *in vitro* by *C. trachomatis* (17). However, as far as we know, this is the first study demonstrating the effect of *C. trachomatis* infection on MMP-9/RECK expression in the context of cervical

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carcinogenesis.

In addition to genital infections, behavior and physiological characteristics such as smoking, parity, oral contraceptives, alcohol and age at first sexual intercourse have been described as factors that influence acquisition of HPV and development of neoplastic lesion (35-38). In this context, we found a higher frequency of smoking patients in the study group when compared to the control group. In fact, a large longitudinal study have reported smoking as a risk factor for CIN3 and invasive cervical carcinoma (38). The induction of epigenetic changes could explain this finding, since it has been shown that smoking increases the risk of methylation of the tumor suppressor gene *CDKN2A* (p16), an epigenetic inactivation strongly associated with the pathogenesis of malignancies (39).

In this study, the frequency of women having had first sexual intercourse at age below 15 years was significantly higher in the study group (44%) when compared to the control group (30%). Our data are in agreement with the literature, once sexual precocity is known to increase susceptibility to the first HPV infection, as well as exposure to the carcinogenic effects of HPV, which is associated with higher risk of CIN3 (35,36,40). On the other hand, CIN1 is associated to recent acquisition of HPV infection, regardless of the age of first sexual intercourse (36).

In conclusion, our results show that higher expression of MMP-9 and lower expression of RECK is associated with high-grade cervical intraepithelial neoplasia. Moreover, we show that MMP-9/RECK imbalance is significantly associated with genital infection by *C. trachomatis* and infection by alpha-9 HPV, which includes HPV16. Genital infection by *C. trachomatis* is also associated with high-grade CIN and SCC, suggesting that infection by *C. trachomatis* could act as a cofactor in HPV-

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mediated cervical carcinogenesis and that the MMP-9/RECK imbalance during cervical inflammation might play an important role in this context.

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Tables

Table 1: Profile of social and demographic characteristics in the study groups

| | Study group | | | | |
| | CIN2/CIN3/SCC | | No neoplasia/CIN1 | | *p-value |
Variable	n (136)	(%)	n (196)	(%)	
Age (years)					0.28
≤ 29	44	(33)	48	(25)	
30-39	48	(35)	79	(40)	
≥ 40	44	(32)	69	(35)	
Age at first sexual intercourse					
≤15	60	(44)	59	(30)	0.008
>15	76	(56)	137	(70)	
Ethnicity					0.78
Black	40	(29)	55	(28)	
White	96	(71)	141	(72)	
Parity					0.003
Multiparous	110	(81)	180	(92)	
Nulliparous	26	(19)	16	(08)	
Smoking					0.001
Smoker or former smoker	50	(37)	41	(21)	
Non-smoker	86	(63)	155	(79)	

*Chi-square test.

Table 2: HPV infection in relation to MMP-9 and RECK expression by immunocytochemistry

|	HPV-negative (ref) n (%)	HPV n (%)	*OR (CI 95%)	HPV 16 n (%)	*OR (CI 95%)	Alpha-9 HPV n (%)	*OR (CI 95%)	Alpha-7 HPV n (%)	*OR (CI 95%)
MMP-9									
Moderate /accentuated	65(44)	109 (67)	**2.5 (1.6-4.0)**	55 (72)	**3.3 (1.8-6.0)**	93(70)	**3.0 (1.8-4.9)**	12 (52)	1.5 (0.6-3.7)
Negative/mild	82(56)	54 (33)		21(27)		39 (30)		11(48)	
RECK									
Moderate /accentuated	63 (41)	41(25)	**0.48 (0.3-0.7)**	20 (26)	**0.5 (0.3-0.9)**	33 (25)	**0.48 (0.3-0.8)**	6 (26)	0.5 (0.2-1.3)
Negative/mild	90 (59)	121 (75)		57(74)		98 (75)		17(74)	

*Odds ratio by multiple logistic regression. Adjustment variables: age, age at first sexual intercourse, ethnicity, education, parity, smoking and genital infections.

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Table 3: Genital infections in relation to MMP-9 and RECK expression by immunocytochemistry, HPV infection and cervical lesions

	*General genital infection			Bacterial vaginosis			*Chlamydia trachomatis*		
	Positive	Negative	**OR (CI 95%)	Positive	Negative	**OR (CI 95%)	Positive	Negative	**OR (CI 95%)
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
MMP-9									
Moderate /accentuated	79 (63)	86 (53)	1.4 (0.8-2.4)	43 (61)	141 (56)	1.3 (0.7-2.2)	25 (76)	130 (54)	**2.6 (1.1-6.6)**
Negative/mild	46 (34)	76 (47)		27 (39)	110 (43)		8 (24)	111 (46)	
RECK									
Moderate /accentuated	40 (31)	65 (40)	0.7 (0.4 -1.3)	23 (32)	88 (34)	1.1 (0.6-2.0)	7 (20)	98 (40)	**0.3 (0.2-0.9)**
Negative/mild	88 (69)	99 (60)		49 (68)	167 (66)		27 (80)	148 (60)	
HPV									
Positive	72 (57)	68 (43)	1.2 (0.6-2.6)	43 (59)	123 (50)	1.3 (0.8-2.3)	17 (55)	111 (46)	1.4 (0.6-3.0)
Negative	54 (43)	91 (57)		30 (41)	124 (50)		14 (45)	130 (54)	
HPV multiple infection									
Yes	26 (36)	21 (31)	1.2 (0.6-2.6)	15 (35)	37 (30)	1.4 (0.6-3.0)	9 (53)	34 (31)	2.7 (0.8-9.0)
No	46 (64)	46 (69)		28 (65)	85 (70)		8 (47)	76 (69)	
Study groups									
CIN2/CIN3/SCC	61 (47)	52 (31)	1.4 (0.8-2.8)	34 (47)	102 (39)	0.9 (0.5-1.8)	19 (56)	82 (33)	**3.7 (1.3-11.3)**
No neoplasia/ CIN 1	69 (53)	115 (69)		39 (53)	157 (61)		15 (44)	168 (77)	

*General genital infections include *Candida* spp, bacterial vaginosis, *Chlamydia trachomatis*, *Neisseria gonorreheae*, *Trichomonas vaginalis*, HSV-1, HSV2 and *Treponema pallidum*.

**Odds ratio by multiple logistic regression. Adjustment variables: age, age at first sexual intercourse, ethnicity, parity and smoking.

Table 4: MMP-9 and RECK expression by immunocytochemistry in relation to cervical lesions

	no neoplasia/ CIN1 n (%)	CIN2/ CIN3/ SCC n(%)	*OR (CI 95%)	**no neoplasia n(%)	CIN1 n(%)	*OR (CI 95%)	CIN2 n(%)	*OR (CI 95%)	CIN3 n (%)	*OR (CI 95%)	SCC n(%)	*OR (CI 95%)
MMP-9 Moderate /accentuated	82 (44)	102 (76)	**4.2 (2.2-7.8)**	74(45)	8 (38)	1.6 (0.5-5.6)	23 (77)	**3.1 (1.0-9.9)**	61(77)	**5.4 (2.4-12.4)**	18 (72)	2.1 (0.5-8.7)
Negative/mild	05 (56)	32 (24)		92(55)	13 (62)		7 (23)		18 (23)		7 (28)	
RECK Moderate /accentuated	85 (44)	26 (20)	**0.4 (0.2-0.7)**	77 (45)	8 (32)	0.7 (0.05-2.4)	3 (10)	**0.2 (0.05-0.84)**	17 (22)	**0.4 (0.2-0.9)**	6 (24)	0.4 (0.1-2.0)
Negative/mild	110 (56)	106 (80)		93 (55)	17 (68)		26(90)		61 (78)		19(76)	

*Odds ratio by multiple logistic regression. Adjustment variables: age, age at first sexual intercourse, ethnicity, education, parity, smoking habit and genital infections.
**Negative cases for cervical lesion were used as reference for individual analysis of CIN1, CIN2, CIN3 and SCC.

Figure Legends:

Figure 1: Electrophoretic analysis of the amplified fragments by multiplex polymerase chain reaction in 8% polyacrylamide gel stained with ethidium bromide. **Lane C_1:** positive control of *T. vaginalis* and HSV-1 (170 and 123 base pairs (bp)); **lane C_2:** positive control of HSV-2, *M. genitalium* and *T. vaginalis* (249, 193 and 170 bp); **lane C_3:** positive control of *C. trachomatis* and *T. pallidum* (361 and 291 bp); **lane C_4:** positive control of *T. pallidum* and HSV-2 (291 and 249 bp); **lane C_5:** positive control of *N. gonorrhoeae* (162 bp); **lane A_1:** positive sample for *C. trachomatis* (361 bp); **lane A_2:** positive sample for *T. vaginalis* (170 bp); **lane A_3:** positive sample for HSV-2 (249 bp); **lane A_4:** positive sample for *T. pallidum* (291 bp); **lane A_5:** positive sample for *N. gonorrhoeae* and HSV-1 (162 and 123 bp); **lane A6:** positive sample for *N. gonorrhoeae* (162 bp); **lane A_7:** positive sample for *T. vaginalis* (170 bp); **lane A_8:** positive sample for *M. genitalium* and *T. vaginalis* (193 and 170 bp); **lane C-:** negative control; **M:** molecular weight marker (25 bp Invitrogen).

Figure 2: mRNA and protein expression of MMP-9 and RECK in cervical samples. **Figures 2A, 2B, 2D and 2E** represent immunocytochemical analysis: A) mild expression of MMP-9 in normal Pap-smear; B) accentuated expression of MMP-9 in CIN3; D) accentuated expression of RECK in normal Pap-smear; E) mild expression of RECK in CIN3 (Nikon ACT-1 1.200C DXM with 400x optical zoom). **Figures 2C and 2F:** graphical representation of gene expression of MMP-9 (C) and RECK (F) in the study groups. *p=0.2; ** p= 0.03

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Figure 1

Figure 2
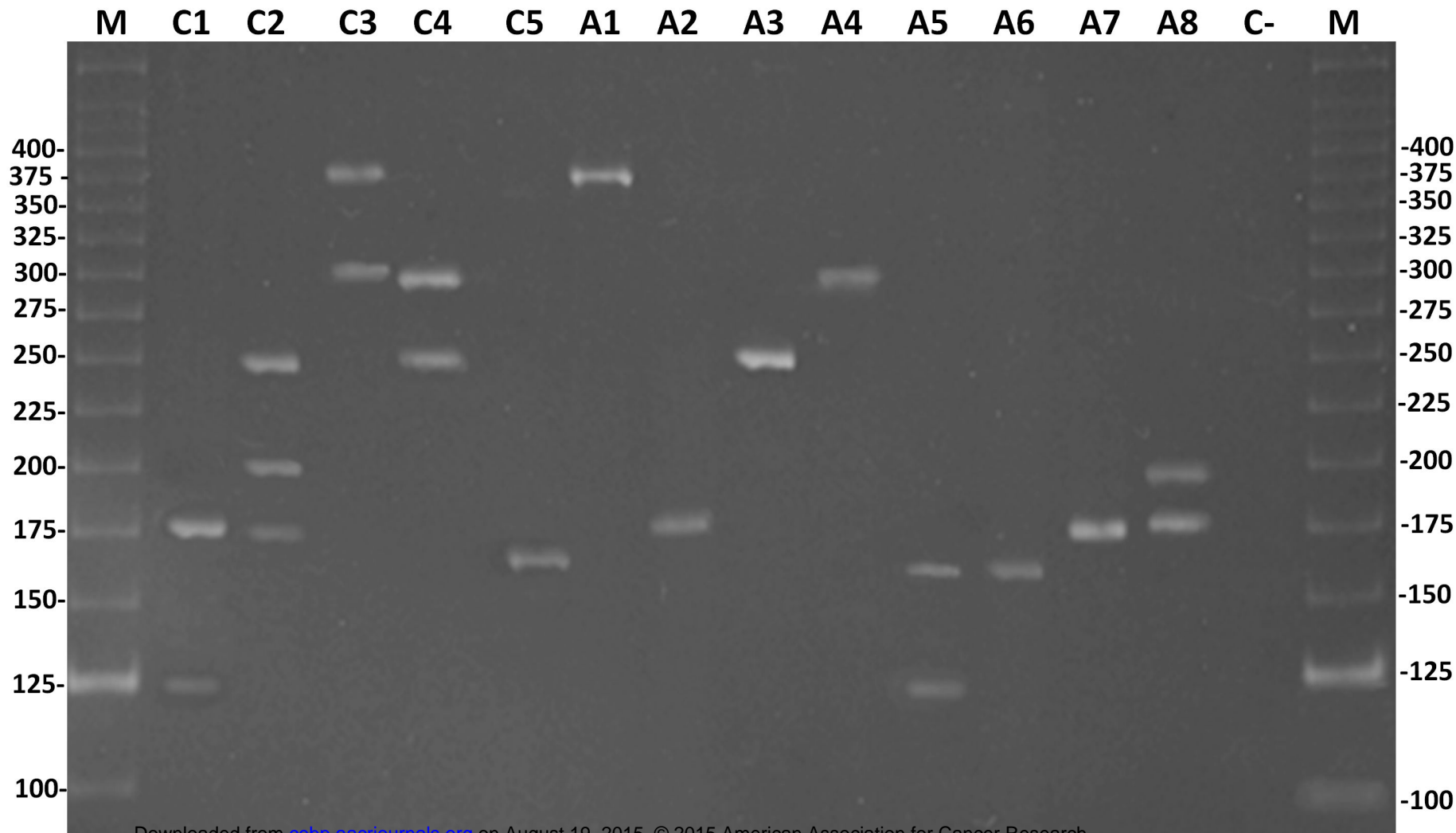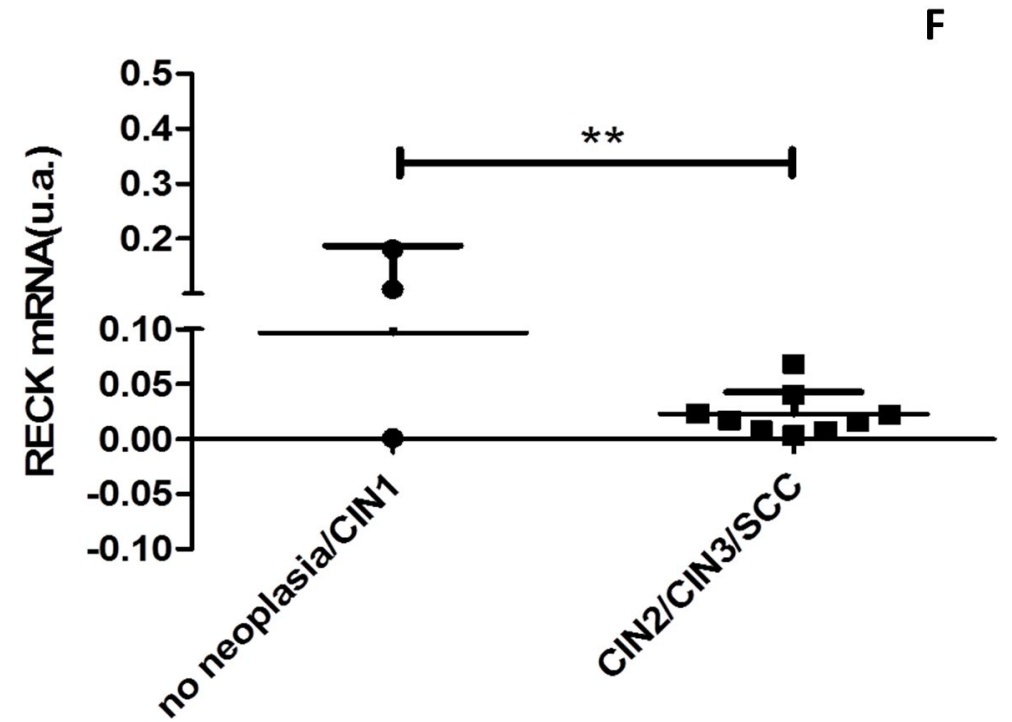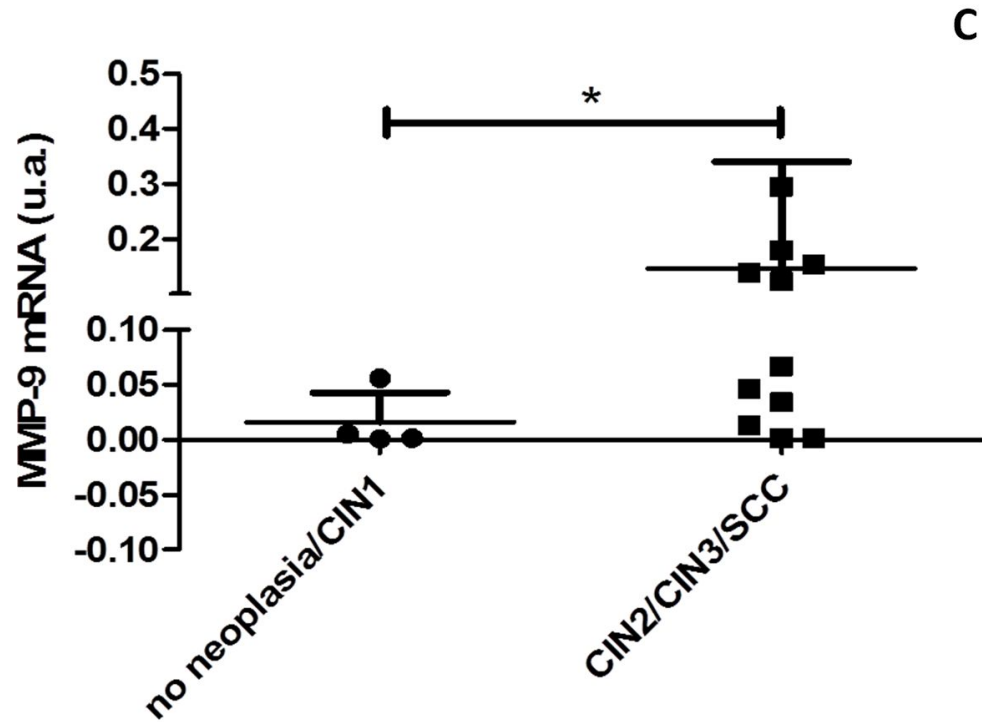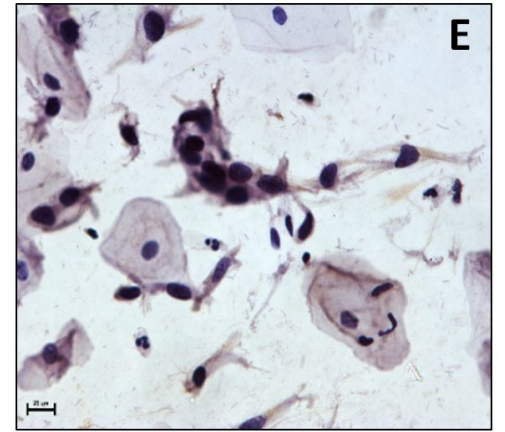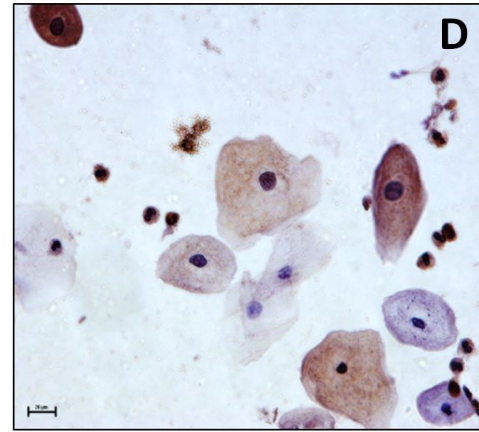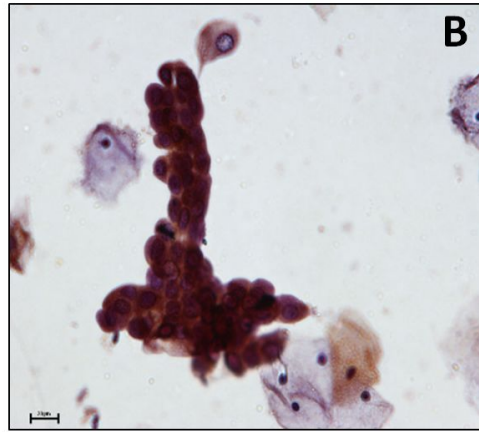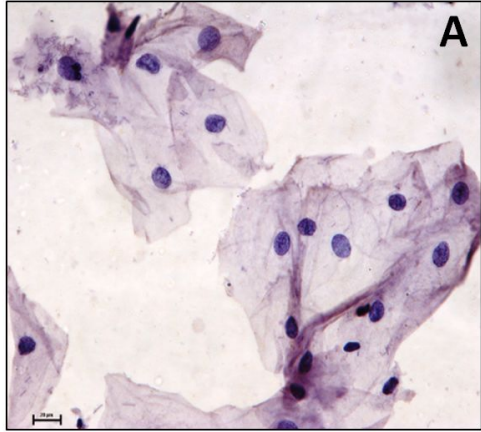
MMP-9

RECK

Cancer Epidemiology, Biomarkers & Prevention

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MMP-9/RECK imbalance: a mechanism associated with high-grade cervical lesions and genital infection by Human Papillomavirus (HPV) and Chlamydia trachomatis

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