JUÇARA MARIA DE CASTRO SOBRINHO

ASSOCIAÇÃO DA COINFECÇÃO POR PAPILOMAVÍRUS HUMANO (HPV) *Chlamydia trachomatis*, VAGINOSE BACTERIANA E RESPOSTA INFLAMATÓRIA COM A GRAVIDADE DA NEOPLASIA CERVICAL

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Tocoginecologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutora em Ciências da Saúde, na área de concentração em Oncologia Ginecológica e Mamária.

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Dedico este trabalho...

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Nada teu exagera ou exclui.
Sê todo em cada coisa.
Põe quanto és no mínimo que fazes.
Assim, em cada lago, a lua toda brilha porque alta vive.

.....

Fernando Pessoa

Sumário

Símbolos, Siglas e Abreviaturas

AN – Padrão anaeróbio (*Standard anaerobic*)

CAISM – Centro de Atenção Integral à Saúde da Mulher

CT – *Chlamydia trachomatis*

CI/ IC – *Confidence intervals* (intervalo de confiança)

95%CI – 95% *confidence intervals*

COX-2 – Ciclo-oxigenase ou *prostaglandin-endoperoxide synthase 2v*

DNA – Ácido desoxirribonucleico (*Deoxyribonucleic acid*)

et al – E outro(s); e outra(s)

EZT – Excisão da zona de transformação

FCM – Faculdade de Ciências Médicas

IL-1 and IL-8 – Interleucina (interleukins)

HPV – Papilomavírus humano (*Human papillomavirus*)

HR-HPV – HPV de alto risco (*High-risk HPV)*

HSV-2 – Herpes vírus tipo 2

HSIL – Lesão intraepitelial escamosa de alto grau (*High-grade squamous intraephitelial lesion*)

IR – *Inflammatory response*

IST/STI – Infecções sexualmente transmissíveis (*sexually transmitted infection*)

IARC – *International Agency for Research on Cancer*

LB – Padrão lactobacilar (*Standard lactobacilli*)

LSIL – Lesão intraepitelial escamosa de baixo grau (*Low-grade squamous intraepithelial lesion*)

e.g. – Por exemplo

NIC/CIN – Neoplasia intraepitelial cervical (*Cervical intraepithelial neoplasia*)

PATRICIA – *Papilloma Trial against Cancer In young Adults*

OR – *Odds ratio*

PCR – Reação em Cadeia da Polimerase (*Polymerase Chain Reaction*)

Ref – Referência (*Reference*)

SC – Padrão escasso não classificável (*Standard scarce unclassifiable*)

STD – *Sexually transmitted disease*

SLPI – *Secretory leukocyte protease inhibito*r

Th1 – Linfócitos T helper

TCD8+ – LinfócitoT Citotóxicos

UNICAMP – Universidade Estadual de Campinas

UCM – *Universal Collection Medium*

VB/BV – Vaginose bacteriana *(bacterial vaginosis*)

ZT – Zona de transformação

Resumo

Objetivo: Analisar a associação entre a coinfecção por papilomavírus humano (HPV), C*hlamydia trachomatis* (CT), vaginose bacteriana (VB) e resposta inflamatória (RI) com a gravidade da neoplasia cervical**. Sujeitos e métodos**: Estudo experimental, de corte transversal, realizado em Campinas, São Paulo e em Goiânia, Goiás, Brasil. A casuística incluiu amostras biológicas de 290 mulheres consecutivas submetidas à excisão da zona de transformação (EZT) ou conização. Para o estudo que avaliou a coinfecção entre HPV e CT e a associação com gravidade da neoplasia cervical foram selecionadas 251 (86,6%) mulheres infectadas por HPV de alto risco (HR-HPV). A detecção de HPV foi realizada utilizando os *primers* PGMY09/11 e a genotipagem através de hibridização reversa em pontos. A detecção da CT foi realizada por polimerase *chain reaction* (PCR) empregando *primers* cujo alvo é uma região de plasmídio críptico, gerando um fragmento de aproximadamente 512 pares de base. Para o estudo que avaliou a VB e resposta inflamatória e a associação destas condições com a gravidade da neoplasia cervical foram selecionadas 211 mulheres infectadas por HR-HPV, com esfregaços cervicais disponíveis para as análises. A presença de 20% ou mais de células indicadoras no esfregaço cervical

corado pelo método de Papanicolaou foi considerada positiva para VB. A resposta inflamatória nos esfregaços cervicais foi avaliada pela contagem do número de neutrófilos. O encontro de 30 ou mais neutrófilos por campo microscópico, observado sob o aumento de 1000x, foi considerado como presença de resposta inflamatória. **Resultados:** A prevalência de CT em mulheres HPV positivas foi de 15,1% (38/251). Foi observada uma associação significativa em mulheres CT negativas, com 30 anos ou mais, e NIC 2 ou pior diagnóstico, mas esta associação não foi observada em mulheres CT positivas. Infecções por HPV 16 e/ou 18 foram detectadas em 50% das mulheres CT negativas com menos de 29 anos e que apresentavam NIC 2 ou pior diagnóstico, e em 19,5% das mulheres CT negativas com NIC 1 ou não neoplásico. Nestas mulheres, a associação entre HPV 16 e/ou 18 e NIC 2 ou pior diagnóstico foi significativa, mas esta associação não foi observada no grupo CT positivo. Resposta inflamatória e VB foram observadas em 43,5% e 46,2% dos esfregaços cervicais de mulheres com NIC 2. Resposta inflamatória e VB foram observados em 64,2% e 32,6% dos esfregaços cervicais de mulheres com diagnóstico histológico de NIC 3. Nestas mulheres, quando infectadas pelos tipos de HPV 16 e/ou 18, foram observadas resposta inflamatória e VB, respectivamente, em 43,1% e 20% dos casos. Resposta inflamatória apresentou associação estatísticamente significante com NIC 2 ou pior diagnóstico em mulheres infectadas pelos tipos de HPV 16 e/ou 18 (OR= 6,70; 95%IC:2,32-19,31) e por outros tipos de HPV (OR=4,90; 95%IC: 1,86-12,89). Associações significativas foram observadas em mulheres com VB e NIC 2 ou pior diagnóstico, infectadas pelos tipos de HPV 16 e/ou 18 (OR= 3,38; 95% IC :1,07-10,64) e por outros tipos de HPV

(OR= 3,38; 95%IC: 1,15-10,01). **Conclusões:** A infecção por CT detectada por PCR não mostrou associação com o aumento do risco para NIC 2 ou pior diagnóstico em mulheres HPV positivas. Em mulheres CT negativas e com menos de 30 anos de idade, os tipos de HPV 16 e/ou 18 estão associados à NIC 2 ou pior diagnóstico, resultado não observado para as mulheres CT positivas. A VB e resposta inflamatória estão associadas à NIC 2 ou pior diagnóstico em mulheres HR-HPV positivas.

Palavras-chave: HPV,*Chlamydia trachomatis*; neoplasia intraepitelial cervical; PCR; câncer do colo do útero; citologia; vaginose bacteriana; inflamação.

Summary

Objective: To analyze the association between co-infection Human Papillomavirus (HPV) and *Chlamydia trachomatis* (CT), bacterial vaginosis (BV) and inflammatory response with the severity of the cervical neoplasia**. Subjects and methods**: This is cross-sectional experimental study carried through in Campinas, São Paulo and in Goiânia, Goiás, Brazil. The casuistic included 290 consecutive women submitted a the Excision of the Transformation Zone or conization due CIN 2 and CIN 3. For the study that evaluated the association between HPV and CT and severity of cervical neoplasia were selected 251 women who were infected with high-risk HPV (HR-HPV). HPV detection .was performed by PCR using primers PGMY09/11 and genotyping by reverse lineblot hybridization assay. The detection of CT was performed by PCR. For the study that evaluated the association between BV and inflammatory response with the severity of cervical neoplasia were selected 211 women infected with HR-HPV with cervical smears available for analysis. The presence of 20% or more clue cells in cervical smears stained by the Papanicolaou method was considered positive for VB. Inflammatory response was assessed by counting the number of neutrophils. The finding of 30 or more neutrophils per field observed under 1000x magnification was taken as presence

of inflammatory response. **Results:** The prevalence of CT in HPV positive women was 15.1% (38/251). Significant association was observed between women with 30 years or older and CIN 2 or worse diagnosis for those CT negative, but this association was not observed for those CT positive. HPV 16 and/or HPV 18 were detected in 50% of the women ≤ 29 years age with CIN 2 or worse diagnosis who were CT negative, and in 19.5% for those women with CIN 1 or no neoplastic in histological diagnostic. In these women the association between HPV 16 and/or 18 and CIN 2 or worse diagnosis was significative, but this association also was not observed considering the CT positive group**.** Inflammatory response and BV were observed in 5.5% and 16.7% of cervical smear of women with no neoplastic diagnosis and were observed in 22.9% and 12.5% of cervical smears of women with CIN 1 in histological diagnosis. Inflammatory response and BV were observed in 43.5% and 46.2% of cervical smears of women with CIN 2 in histological diagnosis. The BV prevalence was higher in cervical smears of women infected by the types 16 and/or 18 (25.6%) and inflammatory response was more observed in cervical smears of women infected by other HPV types (25.6%). Inflammatory response and BV were observed in 64.2% and 32.6% of cervical smears of women with CIN 3 in histological diagnosis and were more observed in cervical smears of women infected types 16 and/or 18 representing respectively 43.1% and 20.0% of cases. Inflammatory response and BV were more observed in cervical smears of women infected types 16 and/or 18 in women with invasive carcinoma, representing 27.2% and 18.2% of cases respectively. Inflammatory response was significantly associated with CIN 2 or worse diagnosis in women

infeted by HPV16 and/or HPV 18 (OR= 6.70; 95%CI : 2.32-19.31) and HPV other types than HPV16 and/or18 (OR=4.90; 95%CI: 1.86-12.89). Significant associations with BV and CIN 2 or worse diagnosis were observed in women infected by HPV 16 and/or 18 (OR= 3.38; 95%C1:07-10.64) and HPV types other than HPV16 and/or 18 (OR=3.38; 95%CI: 1.15-10.01). **Conclusions**: CT infection detected by PCR, does not increase the risk for CIN 2 or worse diagnosis in HPV positive women. HPV 16 and/or HPV 18 types in young women are associated with CIN 2 or worse disgnosis, but without obvious association with CT. BV and inflammatory response are associated with CIN 2 or worse diagnosis in women HR-HPV positives women.

Key words: HPV, *Chlamydia trachomatis;* cervical intraepithelial neoplasia; PCR; uterine cervical neplasms; cytology; bacterial vaginosis; inflammation.

1. Introdução

O câncer do colo uterino, mesmo com campanhas e métodos de diagnósticos eficazes, continua sendo uma importante causa de morte por câncer entre mulheres em países em desenvolvimento. No mundo, morrem anualmente cerca de 230.000 mulheres devido ao câncer do colo de útero, das quais 80% em países em desenvolvimento (1) Este câncer evolui a partir de lesões precursoras, chamadas de neoplasias intraepiteliais cervicais (NIC). Estas lesões, classificadas histologicamente com base na atipia progressiva das células epiteliais (2), são usualmente pequenas e frequentemente aparecem como focos de células atípicas que se misturam ao tecido normal, mas sem constituir uma massa tumoral bem definida (3).

As NIC são caracterizadas pela perda gradual de funções celulares básicas, como divisão e diferenciação. As células anormais perdem gradualmente as funções de controle de crescimento normal, não se diferenciam adequadamente e, como consequência, há proliferação desordenada. Se a anormalidade progride, as células perdem gradativamente a sua capacidade de diferenciação até que toda a espessura do epitélio seja composta por células indiferenciadas (3, 4), A

NIC 1 é caracterizada por atipia nuclear mínima, alterações celulares restritas ao terço inferior do epitélio cervical, baixos índices de mitoses e ausência de mitoses bizarras ou anormais. As NIC 2 e NIC 3 são caracterizadas por pleomorfismo e hipercromasia mais intensa. Além disso, as atipias chegam até o terço médio do epitélio cervical, no caso das NIC 2, ou ocupam o terço superior do epitélio cervical, nas NIC 3 (3).

A infecção genital por HPV é considerada a virose sexualmente transmissível mais comum (5,6). O pico de prevalência ocorre em mulheres com idade em torno de 25 anos, com taxas de infecção variando entre 28% e 46%, e parece diminuir com o aumento da idade. Por outro lado, vários estudos têm mostrado um pico da prevalência da infecção em mulheres abaixo de 25 anos e outro depois dos 55 anos (7). Entretanto, 10% a 20% das mulheres infectadas pelo HPV desenvolvem infecções persistentes e, consequentemente, lesões cervicais (8-10).

Tipos específicos de HPV determinam riscos diferentes para a persistência e a progressão definidas pelo vírus. A infecção por HPV de alto risco oncogênico é uma causa necessária, mas não suficiente para a neoplasia cervical. O risco de carcinoma cervical associa-se principalmente aos HPV 16, 18 e 45 (11). Quando a infecção persiste por dois anos, o HPV 16 possui maior capacidade de causar NIC e câncer do que qualquer outro tipo potencialmente oncogênico (12). Comparativamente, outros tipos apresentam um risco absoluto menor (13). O tipo oncogênico 16 é o mais prevalente na população geral e nas portadoras de lesões precursoras e câncer cervical. Estudos têm revelado que o risco de NIC 3 é maior, considerando os tipos oncogênicos 16 e 33, mas não para os 18 e 45.

O HPV 33 parece ter menor potencial oncogênico em termos de progressão de NIC 3 para carcinoma cervical, quando comparado com os HPV 18 e 45. Por outro lado, o potencial do HPV 33 para induzir NIC 3 parece ser maior do que os potenciais de risco determinados pelos tipos 18 e 45 (8, 4). Ribeiro et al. (14) relataram uma alta prevalência de HPV (86%) em pacientes com diagnóstico de NIC ou carcinoma invasor. Outro dado importante encontrado no estudo foi que a prevalência de HPV 16 aumentou proporcionalmente com a gravidade da lesão.

A infecção por CT é uma doença sexualmente transmissível comum, com a estimativa de aproximadamente 92 milhões de indivíduos infectados a cada ano (15,16). A prevalência geral da infecção é em torno de 15%, podendo variar de 8% a 40% de acordo com a população estudada. As taxas mais altas estão relacionadas a grupos de risco (adolescência, promiscuidade, antecedentes DST, parceiro com uretrite não gonocócica, presença de ectopia cervical e/ou cervicite mucopurulenta) (17).

Este número pode estar ainda subestimado, devido ao alto número de infecções assintomáticas no trato genital inferior de homens e mulheres. Estudos indicam que o número de infecções assintomáticas é muito alto, sendo cerca de 50% em populações de homens e chegando a 80% em mulheres (18-20). Este grande grupo de infectados assintomáticos traz consigo não somente o risco das sequelas de longo tempo, mas também sustentam a transmissão dentro da comunidade (21). Paralelamente, a falta da triagem preventiva da infecção causada por CT em muitos países do mundo contribui para o desconhecimento

da prevalência desta infecção (19). Acredita-se que o atual número de registros de casos represente somente uma fração das populações infectadas (22,23).

Estudos têm demonstrado que infecções sexualmente transmissíveis (IST) parecem atuar em conjunto com a infecção por HPV, acrescendo o risco de neoplasia cervical ou câncer cervical invasivo (24,25). A infecção por CT parece ampliar a suscetibilidade à infecção por HPV em um nível basal, por facilitar o acesso às células epiteliais basais por microabrasões ou por alterar as características das células epiteliais, aumentando a carga viral da infecção e facilitando a persistência. A infecção concorrente por CT pode ainda impedir a resolução da infecção por HPV e inibir a apoptose por bloquear a liberação mitocondrial de citocroma C e de caspase 3, que, por sua vez, permitem que a célula infectada escape do ataque do linfócitoT Citotóxicos (T CD8+) Killer. Tem sido registrado também que a infecção por CT inibe a função natural das células Killer de produzir interferon gama e o desenvolvimento da resposta mediada por linfócitos T helper 1(Th1) (25).

É importante considerar que a infecção por CT está associada à hiperplasia de células de reserva e metaplasia, processos relacionados à carcinogênese cervical (26). De fato, a CT causa uma inflamação grave na cérvice, algumas vezes associada a atipias metaplásicas na zona de transformação (ZT). A prevalência de CT é maior em mulheres com achados anormais na citologia, quando comparadas com as mulheres que apresentam citologia normal (27).

Golijow et al. (24) observaram que as prevalências de DNA de HPV e CT são maiores na neoplasia cervical de alto grau e que mulheres CT positivas

aparentaram ter maior risco de lesão intraepitelial escamosa de baixo e alto graus depois de controladas por idade e pela positividade para DNA de HPV. Neste estudo, o tipo 16 foi predominantemente detectado. Finam et al. (28) demonstraram que as infecções por CT e HPV estão relacionadas com neoplasia intraepitelial cervical. Paba et al. (29) indicaram que a infecção por CT favorece a infecção e persistência de vários tipos de HPV de alto risco oncogênico, inibição da apoptose, superexpressão dos oncogenes virais e transformação celular. Barros et al. (30) demostraram em um estudo que a prevalência de infecção por HPV foi de 86,3% e a soropositividade para CT foi de 26%. Trinta e uma mulheres (27,4%) eram soropositivas para CT e HPV-DNA. O tipo de HPV mais prevalente em mulheres CT soropositivas foi o HPV 16 (51,6%) e este também foi o tipo mais presente nos casos de neoplasias. A positividade para o HPV, especialmente os tipos 16 e 18, e a soropositividade para CT estavam significativamente associadas com o diagnóstico de neoplasia de alto grau.

Existem ainda outros estudos que não encontraram associação entre a infecção HPV e a positividade para CT com a gravidade da neoplasia cervical (31,32). Reesink-Peters et al. (33), mesmo encontrando maior prevalência de anticorpos anticlamidiais em mulheres com câncer cervical invasivo, quando comparadas a mulheres com NIC, verificaram que a prevalência dos anticorpos anticlamidiais não aumentou significativamente com o aumento da gravidade da neoplasia nos dois grupos estudados, sugerindo que é pouco provável que a CT exerça algum papel na progressão de neoplasia cervical.

Mecanismos imunológicos exercem um papel central na etiologia e progressão de muitos cânceres (34,35). A inflamação promove a carcinogênese e a progressão do tumor (36). Adicionalmente, estudos têm demonstrado que os processos inflamatórios podem atuar como cofatores no desenvolvimento de NIC, por possibilitarem a persistência da infecção pelo HPV de alto risco oncogênico e o desenvolvimento e manutenção das lesões intraepiteliais cervicais de alto grau nestas mulheres (37,38). Há indicações de que os macrófagos aumentam linearmente com a progressão da lesão intraepitelial escamosa, migrando do estroma para o epitélio, modulados pela resposta inflamatória local e por células alteradas da zona de transformação (39). Na resposta às infecções crônicas, a inflamação resulta na produção de oxidantes antimicrobianos não específicos, que também podem causar dano ao DNA celular do hospedeiro (38). Acredita-se que as infecções por HPV não evocam uma resposta inflamatória. No entanto, a infecção por CT resulta em maiores níveis de citocinas, o que origina uma resposta inflamatória mais grave (38-40).

A VB é a causa mais frequente de descarga vaginal em mulheres em idade reprodutiva e sexualmente ativas, representando de 9% a 38% dos casos atendidos em clínicas ginecológicas. Há indicações de alta taxa de detecção de VB em mulheres com diagnóstico de lesão intraepitelial de alto grau quando comparadas a mulheres sem anormalidade cervical. Esta condição é de particular interesse, pois frequentemente há coexistência com cervicites e isto constitui um possível fator de risco para a aquisição de infecção por HPV (41). A síndrome é resultante de mudanças na flora vaginal normal, que levam ao aumento de

bactérias anaeróbias estritas e facultativas (*Gardnerella vaginalis, Prevotella* sp*, Bacteroides* sp*, Peptostreptococcus* sp *Mobiluncus* sp e *Atopobium vaginae*) e à diminuição de *Lactobacillus* sp, sendo mais frequente em mulheres em idade reprodutiva. Há uma depleção de lactobacilos produtores de peróxido de hidrogênio favorecendo a sobrevivência de agentes sexualmente transmissíveis no local, conduzindo ao aumento do risco para a infecção por outros agentes (42). De fato, há indicações de que essas anormalidades citológicas cervicais ocorram mais frequentemente em mulheres com desequilíbrio da flora vaginal (43-45).

Considerando o caráter multifatorial envolvido na carcinogênese cervical, ainda existem dúvidas acerca da história natural destas lesões. A importância da coinfecção por HPV e CT ainda é motivo de discussão, bem como as questões relativas ao grau de inflamação e padrão de flora bacteriana associada. Assim, interessa agregar informações sobre a associação da coinfecção por HPV e CT com a gravidade das neoplasias cervicais, bem como avaliar a importância da resposta inflamatória acentuada e anormalidades da flora bacteriana no processo da carcinogênese cervical.

2. Objetivos

2.1. Objetivo Geral

Analisar a associação entre a coinfecção HPV de alto risco oncogênico e *Chlamydia trachomatis,* vaginose bacteriana e resposta inflamatória com a gravidade da neoplasia cervical.

2.2. Objetivos Específicos

- **Artigo1:** Analisar a associação da coinfecção por HPV de alto risco e *Chlamydia trachomatis* com a gravidade da neoplasia cervical.
- **Artigo 2:** Analisar a associação da vaginose bacteriana e da resposta inflamatória em esfregaços cervicais de mulheres infectadas por HPV de alto risco com a gravidade da neoplasia cervical.

3. Publicações

Artigo 1 – Association of co-infection of HPV *and Chlamydia trachomatis* with severity of cervical neoplasia

Artigo a ser submetido à revista Gynecology Oncology

Artigo 2 – Association of bacterial vaginosis and inflammatory response with severity of cervical neoplasia in women with HPV infection

Artigo a ser submetido à revista Cancer Cytopathology

3.1. Artigo 1

The association between a co-infection with HPV and *Chlamydia trachomatis* and the severity of cervical neoplasia

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ABSTRACT

Objective. To analyze the association between high-risk human papillomavirus (HPV) and *Chlamydia trachomatis* (CT) co-infection and the severity of cervical neoplasia.

Methods. This cross-sectional study included 251 HPV-positive women selected from 290 consecutive women submitted to excision of the transformation zone or conization. HPV-DNA was amplified using PGMY09/11 specific primers and genotyping was performed using a reverse line blot hybridization assay. CT was detected by polymerase chain reaction amplification of a sequence in the cryptic plasmid, generating a fragment of 512 base pairs.

Results. Seventy-one cases were diagnosed as CIN 1 or negative for neoplasia and 180 as CIN 2 or worse. The prevalence of CT in HPV-positive women was 15.1% (38/251). In CT-negative women, a significant association was found between age ≥30 years and a diagnosis of CIN 2 or worse; however, this association was not found for CT-positive women. In CT-negative women ≤29 years of age, HPV 16 and/or 18 were detected in 50% of women with a diagnosis of CIN 2 or worse and in 19.5% of women with a diagnosis of CIN 1 or negative for neoplasia (OR=5.83; 95%CI: 2.19-15.57). No such association was found in CT-positive women in this age group or in older women irrespective of CT status.

Conclusions. No association was found between CT infection and a diagnosis of CIN 2 or worse in women with high-risk HPV. In younger, CT-negative women, infection with HPV 16 and/or 18 was associated with a diagnosis of CIN 2 or worse; however, not in older women.

***Key words*:** HPV, *Chlamydia trachomatis,* PCR, uterine cervical neoplasms.

Introduction

Human papillomavirus (HPV), a sexually transmitted deoxyribonucleic acid (DNA) virus, is widely accepted as being the cause of cervical cancer [1,2]. HPV is thought to be the most common sexually transmitted virus. *Chlamydia trachomatis* (CT) is the most common bacterial sexually transmitted infection worldwide [3-5]. The prevalence of both infections is higher in young women [6,7] and factors that are associated with their acquisition, such as younger age and a greater number of sexual partners, are also shared [8]. Co-infection with CT and HPV may affect a woman's risk of developing cervical neoplasia [9-12].

Studies have shown a higher rate of CT infection in HPV-positive women and this association appears to increase the risk of cervical intraepithelial neoplasia (CIN) and cervical cancer [10,12,13]. Paba et al. [10] suggested that CT infection may facilitate the entry and persistence of multiple high-risk HPV (HR-HPV) types in the cervical epithelium. This in turn could lead to viral integration, inhibition of apoptosis and overexpression of E6/E7 oncogenes, and could eventually result in cell transformation. In addition, Barros et al. [12] suggested that positivity for HPV, particularly HPV types 16 and/or 18, combined with seropositivity for CT was significantly associated with a diagnosis of high-grade neoplasia. Borderline significance was observed after adjustment for HPV.

Nevertheless, other studies have failed to find any association between these infections and the severity of cervical neoplasia [8,14-16]. Castle et al. [16] inferred that co-infection with CT did not increase the risk of high-grade cervical neoplasia after controlling for HPV. Safaeian et al. [8] found no association between CT status as

assessed by DNA or serology, and the risk of cervical pre-malignancy after controlling for carcinogenic HPV-positive status.

With these controversies in mind, the present study was designed to analyze the association between co-infection with HPV and CT and the severity of cervical neoplasia. Women testing positive for high-risk HPV who had been submitted to excision of the transformation zone were admitted to the study.

Methods

Selection of cases

This was a cross-sectional study that selected patients from among 290 consecutive women submitted to excision of the transformation zone or conization. Those who agreed to participate in the study and signed the informed consent form were enrolled. The study was approved by the internal review board of the School of Medical Sciences, University of Campinas (UNICAMP). Prior to submitting the patients to colposcopy, a second cervical smear with endocervical sampling was performed, and the residual material was rinsed and stored in 1.0 mL of Universal Collection Medium (QIAGEN Sample & Assay Technologies, QIAGEN Biotechnology Brazil Ltda.) for HPV and CT DNA testing. Women with a diagnosis or suspected diagnosis of a CIN 2 lesion or worse were submitted to excision of the transformation zone or cervical conization in accordance with the current guidelines. Women with a diagnosis of cervical cancer were referred for specialized treatment. Of the 290 women previously selected, 251 (86.6%) were infected with high-risk HPV, and these women constituted the study sample.

Sample Processing and DNA Extraction

Aliquots of 200 mL of the UCM were centrifuged for 10 minutes at 13,000 g. Supernatants were immediately removed and the cellular pellets were split into two parts and stored at -80^{o} C until resuspension in 200 µL of digestion solution (1mM Tris, 200 mg of proteinase K/µL and 0.5% sodium dodecyl sulfate). Digestion was performed at 55^{o} C for 2 hours and was followed by a 5-minute incubation at 95^{o} C to inactivate proteinase K. Nucleic acids were then purified by phenol-chloroform extraction followed by ethanol precipitation. After the DNA pellet had dried, it was dissolved in 100 µL of Tris/EDTA (1 mM and 100 µM, respectively, pH 8.2). The nucleic acids were kept at -80^{o} C until CT detection was performed.

HPV-DNA Testing

HPV DNA was amplified using the PGMY09/11 primers that amplify a 450-bp fragment of the L1 open reading frame. HPV DNA genotyping was performed using a reverse line blot hybridization assay in which the 450-bp PCR amplicon was hybridized to a nylon strip containing immobilized probes [17]. The strip contained 2 levels of β-globin control probes, 18 HR-HPV probes (HR-HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82 and 83) and 9 low-risk HPV probes (low-risk HPV 6, 11, 40, 42, 53, 54, 57, 66 and 84). The 100 µL final volume of the amplification mixture contained 4 mM of $MgCl_2$, 50 mM of KCl, 7.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA, USA), 200 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate and deoxyguanosine triphosphate, 600 mM of deoxyuridine

triphosphate, 100 pmol of each biotinylated PGMY09/PGMY11 primer pool, and 2.5 pmol of each of the 5'-biotinylated β-globin primers, GH20 and PCO4. The amplification profile was: activation of AmpliTaq Gold for 9 minutes at 95°C, denaturation for 1 minute at 95°C, annealing for 1 minute at 55° C and extension at 72° C for 1 minute, for a total of 40 cycles, followed by a 5-minute terminal extension step at 72°C. Amplicons were denatured in 0.4N NaOH. In a reverse-line blot assay, 40 µL of the denatured product were added to 3 mL of hybridization buffer containing the HPV genotypes and 2 concentrations of the β-globin probes, immobilized on nylon strips. Positive hybridization was detected by streptavidin-horseradish peroxidase-mediated color precipitation on the membrane at the probe line. In specimens that were considered HPV-negative, the 2 β-globin lines (high and low copies) either appeared at levels comparable with those of positive controls or were repeated until the criteria for globin positivity were achieved.

Histopathology

The specimens were reviewed according to the World Health Organization criteria and were classified as: negative for neoplasia, CIN 1, CIN 2, CIN 3, invasive squamous cell carcinoma or adenocarcinoma. Other lesions were classified as non-neoplastic, including cervicitis and squamous metaplasia [18].

***Chlamydia trachomatis* detection**

To test the quality of the DNA samples, amplified β-globin was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. *Chlamydia trachomatis*

was detected by PCR amplification of a sequence in the cryptic plasmid, generating a fragment of about 512 bp [19]. The reaction mixture contained 2.5 mM $MgCl_2$, 200 µM dNTPs, 10 pmol of each primer (H1/H2), 1.5 U of Platinum Taq DNA polymerase (Invitrogen Corporation, San Diego, CA, USA) and 2 µL of the sample in a final volume of 20 µL. Reactions were carried out with an initial incubation at 94°C for 5 minutes, followed by 40 cycles of 1 minute at 94°C, 1 minute at 45°C, and 1 minute at 72°C, and a final elongation step of 7 minutes at 72°C. The entire amplified PCR product was analyzed by polyacrylamide gel electrophoresis. *Chlamydia trachomatis* serovar L2 DNA was used as a positive control.

Statistical analysis

The Statistical Analysis System (SAS) software package, version 8.0, was used throughout the analysis. Data were expressed as percentages of the total or as a frequency of HPV-positive and CT cases. Prevalence rates of CT and HPV types were reported for the different categories of histological diagnosis. The Kruskal-Wallis test was used to analyze the women's age. The association between two categorical variables was tested using odds ratios (OR) with their respective 95% confidence intervals (95%CI).

Results

The mean age of the 251 women included in the study was 34.2 years, with a median age of 32 years (range 17 - 75 years). The mean age of the women who tested positive for CT was 32.4 years compared to a mean age of 34.6 years for the women in the CT-

negative group. This difference was not statistically significant (p=0.10). The mean age of the women who tested positive for HPV 16 and/or 18 was 34.6 years, while the mean age of the women infected with other HPV types was 33.8 years, this difference also not being statistically significant (p=0.62). However, the women with a diagnosis of CIN 2 or worse (mean age 35.8 years) were significantly older than those with a diagnosis of CIN 1 or a non-neoplastic diagnosis (30.5 years) (p=0.006). The data on the women's age are not shown as tables.

In the HPV-positive women, the overall prevalence of CT was 15.1% (38/251). The prevalence of CT was higher in women with a diagnosis of CIN 1 or a non-neoplastic diagnosis (19.7%; 14/71) compared to women with a diagnosis of CIN 2 or worse (13.3%; 24/180); however, this difference was not statistically significant (OR= 0.63; 95%CI: 0.29-1.38) (Table 1).

Table 1: The association between the prevalence of *Chlamydia trachomatis* (CT) and the severity of cervical neoplasia in HPV-positive women submitted to excision of the transformation zone

	CIN2 or worse		CIN 1 or negative		Total	OR (95% CI)*
	n	%	n	%		
CT-positive	24	13.3	14	19.7	38 (15.1)	
CT-negative	156	86.7	57	80.3	213 (84.9)	0.63(0.29-1.38)
Total	180 (100)		71 (100)		251 (100)	

CIN: Cervical Intraepithelial Neoplasia, OR: Odds Ratio, CI: Confidence Interval

In women with a diagnosis of CIN 2 or worse, CT was present in 17.9% of those infected with HPV 16 and/or 18 and in 9.8% of those infected with other HPV types, but this difference was not statistically significant (OR = 2.01; 95%CI: 0.84-4.81; p = 0.08). In women with a diagnosis of CIN 1 or a non-neoplastic diagnosis, CT was present in 28.2% of those infected with HPV 16 and/or 18 and in 9.4% of those infected with other HPV types; however, this difference was only of borderline statistical significance (OR= 3.79; 95%CI: 0.95-15) (Table 2).

Table 2: The association between *Chlamydia trachomatis* (CT) infection status, HPV types and histological outcome in women submitted to excision of the transformation zone

Diagnosis	CT	HPV16/18		Other HPV types		OR (95%CI)
		n	%	n	%	
CIN 2 or	Positive	14	17.9	10	9.8	2.01 (0.84-4.81)
worse	Negative	64	82.1	92	90.2	Reference
Total		78	100	102	100	
		HPV16/18		Other HPV Types		
		n	%	n	%	
CIN 1 or	Positive	11	28.2	3	9.4	3.79 (0.95-15.07)
Negative	Negative	28	71.8	29	90.6	Reference
Total		39	100	32	100	

CIN: Cervical Intraepithelial Neoplasia.

In the CT-negative women, a significant association was found between age ≥ 30 years and a diagnosis of CIN 2 or worse; nevertheless, this association was not found in CT-positive women. For CT-negative women ≥30 years of age, the risk of a diagnosis of CIN 2 or worse was twice as high as the risk of a diagnosis of CIN 1 or a non-neoplastic diagnosis (OR = 2.11; 95%CI: 1.13-3.95). A similar analysis conducted in the CT-positive group showed no statistically significant association (OR = 2.03; 95%CI: 0.5-8.23) (Table 3).

Table 3: Association between age group and the severity of cervical neoplasia, according to *Chlamydia trachomatis* status in women submitted to excision of the transformation zone

	Chlamydia trachomatis-positive cases				
Age Group	CIN2 or worse		CIN 1 or Negative		OR (95%CI)*
	n	%	n	%	
≥ 30 years	12	50	5	36	2.03 (0.5 -8.23)
≤ 29 years	12	50	9	64	
	Chlamydia trachomatis-negative cases				
Age Group	CIN2 or worse		CIN 1 and Cervicitis		OR (95%CI)
	N	%	n	%	
≥ 30 years	98	63	25	44	2.11(1.13-3.95)
≤ 29 years	58	37	32	56	

CIN: Cervical Intraepithelial Neoplasia, * OR: Odds Ratio, CI: Confidence Interval

Considering CT-positive women of 30 years of age or more, the prevalence of HPV 16 and/or 18 was 7.2% in those with a diagnosis of CIN 2 or worse and 13.3% in those with a diagnosis of CIN 1 or a non-neoplastic diagnosis; however, this difference was not statistically significant (OR = 0.50; 95%CI: 0.04-6.08). In CT-positive women ≤ 29 years of age, HPV 16 and/or 18 were present in 8.6% of the women with a diagnosis of CIN 2 or worse and in 17.1% of those with a diagnosis of CIN 1 or a non-neoplastic diagnosis; however, this difference was also not statistically significant (OR = 0.28; 95%CI: 0.04-1.98) (Table 4).

Taking into consideration the group of CT-negative women ≥ 30 years of age, the prevalence of HPV 16 and/or 18 was 48.2% in those with a diagnosis of CIN 2 or worse and 43.3% in those with a diagnosis of CIN 1 or a non-neoplastic diagnosis. This difference was not statistically significant (OR = 1.08; 95%CI: 0.45-2.62). Considering the group of CT-negative women ≤ 29 years of age, the prevalence of an HPV 16 and/or 18 infection was 50% in those with a diagnosis of CIN 2 or worse and 19.5% in the women with a diagnosis of CIN 1 or a non-neoplastic diagnosis. In these women, the association between HPV 16 and/or 18 and a diagnosis of CIN 2 or worse resulted in an OR of 5.83, (95%CI: 2.19-15.57) (Table 4).

Table 4: Association between age group, *Chlamydia trachomatis* (CT) status, HPV types and histological diagnosis

Age Group	CT	HPV 16/18	CIN2 or worse		CIN 1 and Negative		OR (95% CI)
			n	%	n	%	
≥30 years	Positive	Positive	8	7.2	4	13.3	0.50 (0.04-6.08)
		Negative	4	3.6	1	3.3	Reference
	Negative	Positive	53	48.2	13	43.3	1.08 (0.45-2.62)
		Negative	45	41.0	12	40	Reference
		Total	110	100	30	100	
≤ 29 years	Positive	Positive	6	8.6%	7	17.1%	0.28 (0.04-1.98)
		Negative	6	8.6%	2	4.9%	Reference
	Negative	Positive	35	50%	8	19.5%	5.83 (2.19-15.57)
		Negative	23	32.9%	24	58.5%	Reference
		Total	70	100	41	100	

CIN: Cervical Intraepithelial Neoplasia. HPV 16/18: HPV 16 and/or HPV 18. OR: Odds Ratio, CI: Confidence Interval

Discussion

Epidemiological studies have shown that other sexually transmitted infectious agents may have an effect on the pathogenesis of cervical cancer; however, knowledge on the specific role of these pathogens in the natural history of HPV infection is limited

[11]. HPV is the most prevalent sexually transmitted viral infection, while CT is the most prevalent sexually transmitted bacterial infection, and co-infection involving both agents is common [8].

This study showed no association between CT infection, as detected by PCR, and a diagnosis of CIN 2 or worse in women with high-risk HPV. In younger, CT-negative women, infection with HPV 16 and/or 18 was associated with a diagnosis of CIN 2 or worse; however, this association was not found in older women. In fact, HPV 16 merits individual consideration, since it is a more potent carcinogen than the other HPV types [20]. Brotherton et al. [20] showed that HPV 16 is more common in young women rather than in older women with high-grade cervical lesions and emphasized that this finding was consistent with all but one out of eighteen studies identified in the literature. Sideri et al. [21] raised the hypothesis of a genotype-specific natural history implicated in the development of cervical cancer precursors: one type, more frequent, HPV16/18 related, developing quickly and early in life; another one, non-16/18 HR-HPV related, developing later, slowly, through low- to high-grade lesions. These findings may explain the results of the present study, which showed a significant association between the severity of cervical neoplasia and HPV 16 and/or 18 infection in CT-negative women $\leq$ 29 years of age, but failed to find a similar association in the case of women $\geq$ 30 years of age.

A positive association between CT, HPV and the detection of cervical neoplasia or invasive cervical cancer has been reported in several studies in which CT was assessed by serology [12, 22,23]. A pooled analysis of case-control studies on invasive cervical cancer published by the International Agency for Research on Cancer (IARC) found a positive association between positivity for CT and invasive cervical cancer in

HPV-positive women [22]. Dahlström et al. [24] conducted a prospective seroepidemiological study and showed that previous exposure to CT, indicated by the presence of positive serum antibodies, increased the woman's risk of cervical cancer (OR = 1.9; 95%CI: 1.5-2.3). Although there is no consensus, the detection of CT serum antibodies seems to be a better measure of cumulative exposure to CT or exposure occurring for several years before the development of cervical neoplasia [8,14,16, 24].

The association between CT, HPV and the detection of cervical intraepithelial neoplasia or invasive cervical cancer has also been reported in some studies in which CT was assessed by PCR or other DNA tests; however, no consensus was reached [25-28]. De Paula et al. [27] reported that although a significant association was found for HPV infection and the precursor lesions of cervical cancer, a significant association could not be established between these lesions and CT or HPV/CT co-infection. According to Safaeain et al. [8], CT may be associated with cervical cancer because it is associated with HPV acquisition by a casual link through common risk factors such as infected partners and sexual behavior or by a causal disruption of the epithelial tissue. These authors consider it unlikely that CT infection affects HPV persistence and progression to cervical premalignancy. Nevertheless, if CT infection plays any role in the etiology of cervical carcinogenesis, a possible mechanism would be by facilitating the entry and promoting the persistence of HR-HPV as a result of chronic inflammation and resistance to apoptosis [8,28].

Considering the long time involved in the development of cervical neoplasia, it is possible that bacterial infection by CT does not persist long enough to be detectable by DNA testing in cervical epithelial neoplasia [8,23,27,29]. In fact, PCR positivity for CT

may indicate an acute or, in rare cases, a chronic infection and this may explain some of the differences in the results found in these studies.

CT-negative women ≥ 30 years of age were more likely to be associated with a diagnosis of CIN 2 or worse than women ≤ 29 years of age. However, no such association was found in the case of CT-positive women and this finding should be considered with caution. The scarcity of cases that tested positive for CT (38/251) constitutes a limitation of this study and may preclude any inference with respect to determining the risk of developing high-grade neoplasia.

Another limitation of the study refers to the fact that the CT genotypes were not analyzed, and they may represent different risks for cervical cancer [30]. In a study conducted in Brazilian women, a heterogeneous pattern of CT infection was found, with the identification of genotypes D, E, F, and K. CT genotyping revealed that genotypes D and E were the most common, as has also been reported in other studies on the diversity of genotypes [19]

In conclusion, this study showed that current CT infection, as detected by PCR, was not associated with a risk of HPV-positive women developing a high-grade lesion. An association was found between HPV 16 and/or 18 infection and high-grade lesions in younger women in whom CT infection was not detected. The lack of consistent evidence of any association between CT infection and the risk of cervical cancer indicates that the treatment of CT is much more relevant for preventing morbidities such as pelvic inflammatory disease and infertility rather than for reducing the risk of a CIN lesion.

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3.2. Artigo 2

Association between bacterial vaginosis, inflammatory response and the severity of cervical neoplasia in HPV-positive women

Running title: Bacterial vaginosis in cervical cancer.

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Condensed abstract:

In this study, BV and inflammatory response were found to be associated with a diagnosis of CIN 2 or worse, emphasizing the importance of the prevention and treatment of these conditions.

Abstract

Background*:** Vaginal infections may affect susceptibility to and clearance of HPV infection and chronic inflammation has been linked to carcinogenesis. This study aimed to evaluate the association between bacterial vaginosis (BV), inflammatory response (IR) with the severity of cervical neoplasia in HPV-infected women. ***Methods: HPV DNA was amplified using PGMY09/11 primers and genotyping was performed using a reverse line blot hybridization assay in 211 cervical samples from women submitted to excision of the transformation zone. The bacterial flora was assessed in Papanicolaou stained smears, and positivity for BV was defined as ≥20% of clue cells. Present inflammatory response was defined as ≥30 neutrophils per field at 1000x magnification. ***Results*:** Inflammatory response and BV were detected in 43.1% and 46.1% of CIN 2 cases and in 64.2% and 32.6% of CIN 3 cases, respectively. Inflammatory response and BV were found, respectively, in 36.3% and 18.1% of cases of invasive carcinoma. Inflammatory response and infection by HPV 16/18 and by other HPV types were significantly associated with CIN 2 or worse diagnosis. Significant associations were found between a CIN 2 or worse diagnosis ,BV, HPV 16/18 and other HPV types. ***Conclusions***: This study suggests that BV and inflammatory response are associated with a diagnosis of CIN 2 or worse diagnosis. Maybe the appropriate management of vaginal inflammatory environment and BV could benefit the clearance of HPV infection.

***Keywords*:** Human Papillomavirus; bacterial vaginosis; cervical smears; inflammatory response; cervical cancer.

Introduction

Infection with high-risk human papillomavirus (HR-HPV) plays a central role in cervical carcinogenesis. Epidemiological and molecular studies have shown that HR-HPV infection is the causal factor in initiating the progressive transformation that leads to cervical intraepithelial neoplasia (CIN) and cervical cancer[1-2]. Susceptibility to HPV acquisition and the capability of the immune system to clear HPV infection could be affected by vaginal infections that disrupt the intricately balanced vaginal ecosystem and its innate protective mechanisms against infection and disease[3-5]. Factors that may play a role in this progression include smoking, contraceptive use, nutrition, and infectious diseases such as bacterial vaginosis (BV)[6,7].

Bacterial vaginosis is associated with high levels of anaerobic microorganisms and their products that can damage the vaginal epithelium, degrade cervical mucus and cleave immunoglobulin A[3-5]**.** This is the most prevalent cause of abnormal vaginal discharge affecting women of reproductive age[6] and it has been associated with sexually transmitted infections (STIs), including infection with HPV and *Chlamydia trachomatis*[7-9] . The magnitude of the association between BV and HPV remains controversial, with conflicting results that range from the complete absence of any association to a clear positive relationship[7,10,11]**.** A recent meta-analysis indicated a positive association between BV and cervical HPV infection[7]. There are indications that BV may play some role in the development of CIN, but no strong consensus has yet been reached[6,12,13].

Inflammation has long been considered a risk factor for cancer at several organ sites[14]. Chronic inflammation has been linked to various steps involved in carcinogenesis, including cell transformation, tumor promotion, survival, proliferation, invasion, potential to trigger angiogenesis, and metastasis[15-17]. Castle et al.[13] considered as findings of

cervicitis 30 or more neutrophils observed in microscopic field in cervical smear, and they observed association between cervicitis and high-grade cervical lesions in women infected with oncogenic HPV. Tjiong et al. [18] reported an association between cervicitis and high-grade cervical lesions in women infected with oncogenic HPV. Therefore, the objective of the present study was to analyze the association of BV and inflammatory response in cervical smears from HPV-infected women analyze the association importance of these conditions with the severity of cervical neoplasia.

Methods

Selection of cases

This was a cross-sectional that selected from 290 consecutive women undergone to excision of the transformation zone or conization who signed the consentment form. This study was approved by Insitucional Review Board of School Medical Sciences of State University of Campinas. Before to undergo the patients to colposcopy, a second cervical smear with endocervical sampling was carried out, and the residual material was rinsed and stored in 1.0mL of Universal Collection Medium (QIAGEN Sample & Assay Technologies, QIAGEN Biotechnology Brazil Ltda) for HPV and DNA testing. Women with diagnosis or suspicion of CIN2 or worse lesion were submitted to excision of transformation zone or cervical conization according to the current guideline. Women with diagnosis of cervical cancer were referred for specialized treatment. Among the 290 women previously selected, 211 (72.8%) were infected by high-risk HPV and had cervical smear available for re-analysis, and this was the study sample. The average age of these 211 women was 34.2 years, the median was 32 years ranging from 25 to 75 years.

Sample Processing and DNA Extraction

Aliquots of 200 mL of the UCM were were taken for HPV PCR and centrifuged for 10 minutes at 13,000 g. The supernatants were immediately removed and the cellular pellets were split and stored at -80^{o} C prior to nucleic acid extraction and HPV detection. The cellular pellets were resuspended in 200 µL of digestion solution (1mM Tris, 200 mg of proteinase K/µL, and 0.5% sodium dodecyl sulfate) and incubated at 55^{o} C for 2 hours. Digestion was followed by a 5-minute incubation at 95^{o} C to inactivate proteinase K. Nucleic acids were purified by phenol-chloroform extraction followed by ethanol precipitation. After the DNA pellet had dried, it was dissolved in 100 µL of Tris/EDTA (1mM and 100 µM, respectively, pH 8.2). The nucleic acids were stored at -80^{o} C .

HPV-DNA Testing

HPV DNA was amplified using the PGMY09/11 primers that amplify a 450-bp fragment of the L1 open reading frame. HPV DNA genotyping was performed using a reverse line blot hybridization assay in which the 450-bp PCR amplicon is hybridized to a nylon strip containing immobilized probes [12]. The strip contained 2 levels of β-globin control probes, 18 HR-HPV probes (HR-HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82 and 83) and 9 low-risk HPV probes (low-risk HPV 6, 11, 40, 42, 53, 54, 57, 66 and 84). The 100 µL volume of the amplification mixture contained 4 mM of $MgCl_2$, 50 mM of KCl, 7.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA, USA), 200 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate and deoxyguanosine triphosphate, 600 mM of deoxyuridine triphosphate, 100 pmol of each biotinylated PGMY09/PGMY11 primer pool, and 2.5 pmol of each of

the 5'biotinylated β-globin primers, GH20 and PCO4. The following amplification profile was used: activation of AmpliTaq Gold for 9 minutes at 95°C, denaturation for 1 minute at 95°C, annealing for 1 minute at 55° C and extension at 72° C for 1 minute, for a total of 40 cycles, followed by a 5-minute terminal extension step at 72°C. Amplicons were denatured in 0.4N NaOH. In a reverse-line blot assay, 40 μL of the denatured product were added to 3 mL of hybridization buffer containing the HPV genotypes and 2 concentrations of the β-globin probes, immobilized on nylon strips. Positive hybridization was detected by streptavidin-horseradish peroxidase-mediated color precipitation on the membrane at the probe line. In specimens that were considered HPV-negative, the 2 β-globin lines (high and low copies) either appeared at levels comparable with those of positive controls, or were repeated until the criteria for globin positivity were achieved.

Histopathology

The specimens were reviewed according to the World Health Organization criteria [18] and were classified as: CIN 1, CIN 2, CIN 3, invasive squamous cell carcinoma or adenocarcinoma. Other lesions were classified as non-neoplastic, including cervicitis and squamous metaplasia[19].

Evaluation of the pattern of bacterial flora

The bacterial flora was assessed in Papanicolaou stained cervical smears and classified according to the patterns defined by Schnadig et al[20]. The bacterial flora detected in the Pap-stained cervical smears was classified into the following patterns:

- Large bacillus (LB): a pattern characterized by a predominance of *Lactobacilli*.

- Anaerobic (AN): characterized by a wide variation in the bacterial content and absence of bacteria of *Lactobacillus* morphotypes, while occurrence of clue cells may or may not be observed.
- Scanty/ unclassifiable (SC): a pattern characteristic of older women, menopause or amenorrhea/anovulation, in which bacteria are apparently absent.

For the purposes of this study, BV was defined as a change in vaginal microflora from predominately *Lactobacilli* to predominately anaerobic bacteria not normally present in high numbers. Discacciati et al.[12] reported that the presence of 20% or more of indicator cells in a Pap-stained cervical smear represents a sensitive and reproducible criterion for a diagnosis of BV and can be used to diagnose this condition. These authors used Gram-stained smears evaluated according to the criteria defined by Nugent et al. [21] as the gold standard.

In this study, 20 representative fields containing at least 10 epithelial cells were randomly selected and examined under 40X magnification. The smear was considered positive for BV when at least two clue cells were found per field according Discacciati et al.[12] The identification of clue cells was made by examining the edges of the cells. A normal squamous cell has sharp, clear, linear edges, whereas a clue cell has granular, cloudy, rough edges. Stippling over the cytoplasm of a squamous cell didn´t make it a clue cell [21].

Assessment of the degree of inflammation

Cervical inflammation was assessed by counting the number of neutrophils observed in microscope fields on Papanicolaou stained cervical smears from each study subject. Initially, 400x magnification was used to identify cervical mucus in microscopic fields not associated with epithelial cells. Valid fields were observed at 1000X magnification to

identify neutrophils,(identifiable by their multilobed nucle) and the number of neutrophils. Taking as reference the score proposed by Castle et al.[13], presence of inflammatory response was defined as $\geq$30 neutrophils per field. Five non-adjacent fields were evaluated.

Statistical analysis

The Statistical Analysis System (SAS) software program, version 8.0, was used throughout the analysis. Data were expressed as a percentage of the total or as a frequency of HPV-positive, inflammatory response and VB cases. The prevalence rates of VB, inflammatory response and HPV-DNA types were reported for each group of diagnosis. The association of HPV, HPV 16 and/or HPV 18, other types of HPV infection and positivity for BV, and inflammatory response with the final diagnosis was calculated using odds ratios (OR) with their respective 95% confidence intervals (95%CI).

Results

BV and inflammatory response were found in 16.7% and 5.5% of the cervical smears from women with no neoplasia and in 22.9% and 12.5% of women with CIN 1. Considering the cervical smears from women with a histological diagnosis of CIN 2, inflammatory response was found in 43.6% and BV in 46.2% of the smears. Inflammatory response and BV were found in 64.2% and 32.6% of cervical smears from women with a histological diagnosis of CIN 3 and in 36.4% and 18.2% of cases of cases of invasive carcinoma (Table 1).

Among women infected by HPV types 16/18, inflammatory response was significantly associated with CIN 2 or worse diagnosis (OR: 6.70; 95%CI: 2.32-19.31);

among those women infected by other HPV types, inflammatory response also was significantly associated CIN 2 or worse diagnosis (OR: 5.06; 95%CI: 1.93-13.28) (Table 2).

Among women infected by HPV types 16/18, BV was significantly associated with CIN 2 or worse diagnosis (OR: 3.38; 95%CI:1.07-10.64); among those women infected by other HPV types, BV also was significantly associated with CIN 2 or worse diagnosis (OR: 3.30; 95%CI: 1.12-9.74) (Table 2).

Discussion

This study showed that BV and the inflammatory response were associated with of CIN 2 or worse diagnosis, independently if the women were infected by HPV 16/18 or others. Previous report of genital conditions, including nonspecific genital infections/sores or vaginal discharge associated with cervical cancer, suggested a possible link between either genital tract inflammation or changes in bacterial flora consistent with bacterial vaginosis (BV) and cervical cancer [13].

Several hypotheses have been put forward in support of the association between BV and HPV. In BV-negative women, hydrogen peroxide-producing lactobacilli dominate the vaginal microflora and form part of the principal defense mechanisms [8]. Loss of these protective microorganisms and other changes in the vaginal milieu associated with BV may facilitate the survival of other sexually transmitted agents and constitute risk factors for developing vaginal infections. BV has been associated with a reduction in vaginal fluid levels of secretory leukocyte protease inhibitor (SLPI). SLPI is able to block viral infections in vitro[22] and its reduction contributes towards propagating viral replication and vaginal shedding, thereby further enhancing spread of the viruses[23].

Another hypothesis proposes that mucin-degrading enzymes are increased in the vaginal fluid of women with BV. These enzymes, including sialidases, play a role in the degradation of the gel layer coating the cervical epithelium, causing micro-abrasions or alterations in the epithelial cells. Briselden et al.[23] reported positivity for sialidases in 84% of BV-positive women. [7] These enzymes may promote virulence by destroying the protective mucosa barrier, thus increasing susceptibility to cervical HPV.

It is also possible that BV may be a cofactor involved in the acquisition or reactivation of HPV infection by affecting the immunological balance within the cervical tissue as a result of changes in the production of factors such as cytokines, interleukin (IL)ß, IL-10)[24]. Mucosal immune system activation represents a critical response against microorganisms colonizing the reproductive tract. Neutrophil recruitment and activation is considered the main innate immune response against microbial and viral infections of the vaginal mucosa [24].

A meta-analysis found a positive association between BV and cervical HPV infection[7]. Watts et al.[25] noticed that BV was associated with an increased risk of prevalent and incident HPV infection but not with the duration of HPV infection or with the development of squamous intraepithelial lesion (SIL). The association between BV and HPV persisted even after adjustment for the number of lifetime partners and number of current male sex partners, which suggests that the association is not simply the result of shared risk factors for acquisition[26-28] .

On the other hand, a cross-sectional study of women in Costa Rica failed to find any increase in the rate of BV in women with HPV compared to women without HPV [13] .A small prospective study conducted with adolescents did not find that a history of BV, diagnosed by nonstandard criteria, was a risk factor for incident HPV infection[29].

Furthermore, there are indications that BV may play a role in the severity of cervical neoplasia, since abnormal vaginal flora is able to produce nitrosamines that may act as a cofactor in carcinogenesis. In fact, previous reports of genital conditions, such as nonspecific genital infection/sore or vaginal discharge associated with cervical cancer, suggest a possible link between either genital tract inflammation or changes in bacteria flora consistent with BV and cervical cancer[13]. Nam et al.[6] showed that the incidence of CIN was significantly higher in women with BV; however, no statistically significant association was found between BV and CIN in a multivariate analysis. Boyle et al. [30] also reported that women with BV were not more prone to CIN than women without BV. On the other hand, Platz-Christensens et al.[31] showed an association between BV and CIN. Although Peters et al.[32] did not find any association between between BV and CIN, Discacciati et al.[12] showed that BV tended to be more common in women with high-grade squamous intraepithelial lesion than in women with no cytological abnormalities.

Inflammation is a relatively nonspecific physiological response to tissue injury caused by exogenous factors such as microbial infections or chemical irritants [33] . Hallmarks of the inflammatory response include the migration of natural killer cells and phagocytes (e.g. neutrophils and macrophages) that release inflammatory mediators (e.g. IL-1 and IL-8). Inflammation, often in response to chronic infection, results in the production of nonspecific protective antimicrobial oxidants that may also cause oxidative damage to host DNA, leading to cancer [34].

The association of inflammation with many cancers suggests that inflammation may be a universal risk factor for carcinogenesis. HPV infection of the cervix is not believed to be inflammatory[34]. Nevertheless, there is some epidemiological evidence, albeit weak, to suggest that inflammation may be linked to cervical cancer, perhaps as an

HPV cofactor[35,36]. Furthermore, there is an ecological association of greater levels of cervical inflammation in populations with a higher incidence of cervical neoplasia. In Guanacaste, Costa Rica, where rates of cervical cancer are high, unusually high levels of unexplained cervical inflammation have also been reported[13].

Castle et al. [13] noticed that high-grade lesions were associated with a 2-fold increase in the risk of increasing levels of cervical inflammation. These authors suggested that cervical inflammation may contribute to the progression of HPV infections to high-grade lesions. In combination with the high prevalence of cervical inflammation in Guanacaste, Costa Rica, this association suggests that cervical inflammation may be an important contributor to the incidence of high-grade lesions.

Another study developed by Castle et al. [14] focused on examining the potential role of these less well-known cofactors in the outcome of HPV infections. Co-infections may act through direct genotoxicity; however, the most likely biological mechanism may be the induction of inflammation at the cervix leading to genotoxic damage via reactive oxidative metabolites. Inflammation has long been considered a risk factor for cancer at several organ sites. Chronic inflammation results in increased production of reactive oxygen species and a decrease in cell-mediated immunity, two factors that appear to influence the progression of viral infections [37].

An expanding body of literature suggests that cervical carcinogenesis is also associated with inflammation. High rates of cervical cancer often coincide with endemic and epidemic cervicitis. Castle et al.[13] showed an association of cervicitis with high-grade cervical lesions in women infected with oncogenic HPV. In another study, Kulkarni et al.[38] reported an increase in COX-2 expression in human cervical cancer, suggesting that inflammation is linked to cervical carcinogenesis.

In summary, the current study suggests that bacterial vaginosis and inflammatory response are associated with high-grade cervical neoplasia, emphasizing the importance of prevention and successful treatment of BV and cervical inflammation.

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Table 1. Histological outcome in women HPV positives regarding bacterial vaginosis (BV) and inflammatory response (IR)

	Negative		CIN 1		CIN 2		CIN 3		Invasive Carcinoma	
	IR +	BV+	IR +	BV+	IR+	BV+	IR +	BV+	IR +	BV+
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
HPV 16/18	0 (0.0)	1 (5.6)	5 (10.4)	3 (6.25)	7 (17.9)	10 (25.6)	41 (43.1)	19 (20.0)	3 (27.2)	2 (18.2)
HPV types other than 16 and 18	1 (5.5)	2 (11.1)	6 (12.5)	3 (6.25)	10 (25.6)	8 (20.6)	20 (21.2)	12 (12.6)	1 (9.2)	0 (0.0)
Positive cases/Total	1/18 (5.5)	3/18 (16.7)	11/48 (22.9)	6/48 (12.5)	17/39 (43.5)	18/39 (46.2)	61 /95 (64.2)	31/95 (32.6)	4/11 (36.4)	2/11 (18.2)

CIN: Cervical intraepithelial neoplasia; (BV)bacterial vaginosis;(IR) inflammatory response

Table 2. Association between inflammatory response and BV and severity of cervical neoplasia in women infected by HPV 16/18 and HPV types other thar 16/18

	CIN 2 or worse diagnosis	CIN 1 or negative for neoplasia	OR(95%CI)	*p*-value*
HPV 16/18				
IR+	50	5	6.70 (2.32-19.31)	0.0001
IR-	35	23	Reference	
BV+	31	4	3.38(1.07-10.64)	0.027
BV-	54	24	Reference	
HPV types other than 16/18				
IR+	32	7	5.06 (1.93-13.28)	0.00028
IR-	28	31	Reference	
BV+	20	5	3.30 (1.12-9.74)	0.013
BV-	40	33		

CIN – cervical intraepithelial neoplasia, BV- bacterial vaginosis,IR- inflammatory response OR: Odds Ratio, CI: Confidence interval, * Fisher Exact Test

4. Discussão

O propósito deste estudo foi avaliar potenciais cofatores em mulheres infectadas por HR-HPV e, devido ao próprio critério de inclusão utilizado, mulheres com maior chance de serem portadoras de neoplasias cervicais de maior gravidade. Foram avaliadas a importância da coinfecção por CT e as influências da VB e do grau de inflamação como possíveis cofatores na carcinogênese cervical. Os resultados deste estudo demonstraram que a VB e a resposta inflamatória apresentam associação significativa com NIC 2 ou lesão mais grave em mulheres infectadas por HR-HPV. Entretanto, não houve associação significativa entre infecção por CT e gravidade da neoplasia cervical.

Sabe-se que os tipos oncogênicos do HPV são considerados necessários para o desenvolvimento do câncer cervical e suas lesões precursoras (2,3,5). Todavia, este fator não é, por si só, suficiente para completar toda a via causal, necessitando da interação com vários outros cofatores (46). De fato, a etiologia do câncer cervical vem sendo coerentemente descrita e inclui a presença de um grupo limitado de tipos de HPV, como agentes etiológicos, e adicionalmente alguns cofatores que podem promover a persistência ou aumentar a oncogenicidade

do vírus. A CT tem sido considerada um destes cofatores (47). Alguns estudos têm investigado a influência de fatores comportamentais na aquisição, persistência da infecção por HPV e desenvolvimento das lesões (48,49), enquanto outros têm registrado a influência de infecções genitais, inclusive da CT. Um estudo realizado pelo grupo de estudo *Papilloma trial against cancer in young adults* (PATRICIA) (50) registra que infecções pregressas por micro-organismos sexualmente transmissíveis aumenta pouco o risco para o desenvolvimento do câncer . O estudo também demonstra que existem variações destes riscos em populações geograficamente diversas.

Delucca et al. (51) indicaram que a infecção por CT pode ter um papel importante na etiologia da carcingênese cervical por facilitar a entrada e a persistência do HR-HPV. Os autores registraram que isto é próprio da inflamação crônica induzida pela bactéria e da resistência à apoptose, que a infecção persistente por CT parece provocar. Neste trabalho, os autores encontraram pequena associação entre infecção concomitante por CT e HPV. Entretanto, devido ao desenho de corte transversal do estudo, não foi possível determinar a infecção primária nas pacientes com infecções concomitantes por HPV e CT. A detecção de CT e HPV por PCR indica preferencialmente uma infecção aguda e, mais raramente crônica, por CT e uma infecção transitória ou persistente de infecção por HPV.

Por outro lado, vários estudos têm demonstrado uma associação entre a positividade para HPV de alto risco oncogênico, soropositividade para CT e gravidade da neoplasia cervical (16,18,22,24). De fato, os resultados de um estudo

multicêntrico da *International Agency for Reseach and Cancer (IARC)* mostraram o risco aumentado de 2 vezes para o câncer do colo do útero em presença de anticorpos para a CT (OR = 2,1;95% IC:1,1-4,0). É provável que o aumento do risco associado com CT resulte em parte da indução da inflamação no colo do útero devido a danos genotóxicos via metabólitos oxidativos reativos (52). Os mesmos autores concluem que as evidências dessas associações devem ser interpretadas com cautela devido às limitações dos métodos utilizados para medir a exposição. Por outro lado, há estudos que não encontraram asssociação entre a soropositividade para CT em mulheres HPV positivas e a gravidade da neoplasia cervical (24-30).

Golijow et al.(24) hipotetizaram que a sorologia para CT seria uma melhor medida de exposição anterior e que refletiria uma melhor medida de risco em mulheres HPV positivas. Analogamente, uma infecção crônica por CT detectada por PCR nestas mulheres também pode relacionar-se à gravidade da neoplasia cervical por refletir uma exposição anterior e persistente.

Clarke et al. (53) relataram associação positiva entre o pH vaginal e positividade do HPV em um grande estudo de coorte em mulheres selecionadas de forma aleatória. Os autores demonstraram que a alteração do pH vaginal está relacionada com inflamação do trato genital e alteração da flora bacteriana vaginal, ambos sugeridos como cofatores para a persitência do HPV.

Rodriguez-Cerdeira et al.(54) sugeriram uma associação positiva entre VB e HR-HPV. Vários estudos registram que a VB é a principal causa de

corrimento anormal em mulheres em idade reprodutiva, sendo notadamente considerada a condição mais prevalente. Ocorre em mais de 30% da população, embora apresente causa desconhecida. Está substancialmente associada a morbidades em certas populações de pacientes, aumentando a susceptibilidade às infecções sexualmente transmissíveis (16,31,33,35,52). Livengood et al. (55) registraram que 84% das mulheres com diagnóstico positivo para VB também apresentavam resultados positivos para sialidases. Estas enzimas podem aumentar a virlulência por promover a destruição das barreiras de muco, aumentando assim a susceptibilidade à infecção cervical por HR-HPV. Desta forma, a VB poderia facilitar a aderência, invasão e eventualmente a incorporação dos oncogens ativados do HPV dentro do genoma das células da ZT.

A microflora vaginal anormal também está envolvida com a manutenção das infecções subclinicas por HR-HPV. As bactérias anaeróbicas envolvidas na patogênese da VB podem potencialmente alterar os sinais imunológicos e promover a degradação de fatores protetores do hospedeiro, tornando a mucosa mais susceptível à infecção por HR-HPV (56). Um estudo envolvendo a análise citológica de 17.391 esfregaços no Brasil demonstrou que a VB foi frequentemente encontrada em mulheres com infecção por HR-HPV quando comparada com o grupo-controle. Entretanto, ainda não se sabe em que ponto as infecções se correlacionam, pois existe uma forte associação simbiótica entre estas, sendo que ambas ocorrem com muita frequência em mulheres sexualmente ativas (57).

A inflamação é uma resposta fisiológica relativamente inespecífica à lesão de tecidos causada por fatores exógenos, como infecções microbianas ou

irritantes químicos. Alterações inflamatórias não específicas também têm sido relacionadas a aumentos de risco de lesões cervicais pré-neoplásicas entre as mulheres HPV positivas (58,59).

Está muito bem estabelecido que a inflamação crônica aumenta o risco de câncer, e as inflamações subclínicas, geralmente não detectadas, podem ser um importante fator de risco para o câncer. A inflamação crônica aumenta a produção de espécies de oxigênio reativo e diminui a imunidade mediada por células, dois fatores que parecem influenciar a progressão das infecções virais para câncer (34,60,61). Um corpo crescente da literatura sugere que a carcinogênese cervical também está associada à inflamação. Há indicações de que o microambiente gerado pela inflamação é um componente essencial para todos os tumores, mesmo para aqueles em que a relação causal direta com a inflamação não tenha sido comprovada (62).

O presente trabalho sugere que a triagem e o diagnóstico das infecções genitais podem revelar a presença simultânea de diferentes micro-organismos sexualmente transmissíveis. É importante destacar que a prevenção é importante não apenas por evitar ISTs e suas sequelas, mas também por reduzir a influência de infecções concomitantes com a infecção por HR-HPV. Em resumo, são vários os cofatores envolvidos nos processos multicausal e multifatorial da carcinogênese. Ainda não existem dados completamente consensuais a respeito da importância e verdadeira interação entre estes, porém faz-se necessário conhecer e tentar minimizar seus efeitos sinérgicos, principalmente em relação à inflamação e à microbiota vaginal.

5. Conclusões

A prevalência de CT foi maior em mulheres com diagnóstico não neoplásico e com NIC 1 do que em mulheres com NIC 2 ou pior diagnóstico, mas esta diferença não foi estatisticamente significante.

Houve maior positividade para CT em mulheres com diagnóstico não neoplásico e com NIC 1, infectadas pelos tipos 16 e/ou 18, mas esta diferença também não foi estatisticamente significante.

A positividade para CT foi mais frequente em mulheres com NIC 2 ou pior diagnóstico infectadas pelos HPV 16 e/ou 18 do que nas mulheres infectadas por outros tipos de HPV, mas esta diferença não foi estatisticamente significante.

Nas mulheres CT negativas, uma associação estatisticamente significante foi observada entre mulheres com idade maior ou igual a 30 anos e o diagnóstico de NIC 2 ou lesão mais grave, mas esta associação não foi observada em mulheres HPV positivas. Análises similares foram realizadas considerando as mulheres HPV positivas, mas os resultados não apresentaram significância estatística.

Infecções por HPV 16 e/ou 18 foram detectadas em 50% das mulheres CT negativas com idade menor ou igual a 29 anos, e que apresentavam NIC 2 ou

pior diagnóstico, e em 19,5% das mulheres CT negativas com NIC 1 ou não neoplásico. Nestas mulheres a associação entre HPV 16 e/ou 18 e NIC 2 ou pior diagnóstico foi significativa, mas não observada no grupo CT positivo.

Este estudo mostrou uma associação significativa entre a gravidade da neoplasia cervical, infecção por HPV16 e/ou 18 em mulheres CT-negativas com idade menor ou igual a 29 anos, quando comparada com os outros tipos de HPV, mas não demonstrou associação semelhante em mulheres com 30 anos ou mais.

Vaginose bacteriana e resposta inflamatória mostraram associação significativa com NIC 2 ou pior diagnóstico em mulheres infetadas pelos HPV 16 e/ou 18 ou por outros tipos de HPV.

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7. Anexos

7.1. Anexo 1 – Parecer do CEP

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA

www.fcm.unicamp.br/pesquisa/etica/index.html

CEP, 21/12/10
(Grupo III)

PARECER CEP: Nº 1205/2010 (Este nº deve ser citado nas correspondências referente a este projeto).
CAAE: 0936.0.146.000-10

I - IDENTIFICAÇÃO:

PROJETO: **"RELAÇÃO ENTRE A CO-INFECÇÃO POR PAPILOMAVÍRUS HUMANO, CHLAMYDIA TRACHOMATIS, INFLAMAÇÃO E VAGINOSE BACTERIANA COM A GRAVIDADE DA NEOPLASIA CERVICAL"**
PESQUISADOR RESPONSÁVEL: Juçara Maria de Castro Sobrinho
INSTITUIÇÃO: CAISM / UNICAMP
APRESENTAÇÃO AO CEP: 06/12/2010
APRESENTAR RELATÓRIO EM: 21/12/11 (O formulário encontra-se no *site* acima).

II - OBJETIVOS

Analisar a associação entre a co-infecção pelo vírus do HPV e da Chlamydia trachomatis, das anormalidades em flora bacteriana e do grau de inflamação com a gravidade da neoplasia cervical, além de avaliar a importância da idade.

Determinar a prevalência de co-infecção do HPV/Chlamydia trachomatis em mulheres com diagnóstico de neoplasia intra-epitelial cervical comprovada pela biópsia. Avaliar se há associação entre a co-infecção HPV/Chlamidia trachomatis, o grau de inflamação, a presença de vaginose bacteriana e a gravidade das neoplasias intraepiteliais.

III - SUMÁRIO

Há evidência epidemiológica que sugere que o Vírus do Papiloma Humano (HPV) e a Chlamydia trachomatis desempenham um papel central na etiologia da neoplasia intra-epitelial cervical (NIC). Processos inflamatórios podem atuar como co-fatores no desenvolvimento de NIC por possibilitarem a persistência da infecção pelo HPV de alto risco oncogênico e o desenvolvimento e manutenção das lesões intra-epiteliais cervicais de alto grau (LIEAG) em mulheres infectadas com HPV. A taxa de detecção de vaginose bacteriana (VB) em mulheres com diagnóstico de LIEAG é alta quando comparadas a mulheres sem nenhuma anormalidade citológica. VB freqüentemente coexiste com cervicites e constitui um possível fator de risco para a aquisição de infecção por HPV. O objetivo deste estudo é analisar a associação entre a co-infecção Vírus do Papiloma Humano/Chlamydia trachomatis (HPV/CT), anormalidades em flora bacteriana e grau de inflamação com a gravidade da neoplasia cervical. Para este estudo retrospectivo de corte transversal, será utilizado material biológico de 260 mulheres com diferentes graus de NIC e cervicite com resultados de detecção e genotipagem de HPV. A pesquisa de Chlamydia trachomatis será realizada por PCR empregando primers cujo alvo é uma região de plasmídio críptico de 7512 pares de base. Esfregaços cervicais colhidos no momento da colposcopia serão utilizados para análise do grau de inflamação e padrão de flora bacteriana. A análise dos resultados será realizada utilizando-se o teste qui-quadrado para testar associações

entre as variáveis e para estimar a magnitude da associação serão calculados valores de odds ratio (OR) com respectivos intervalos de confiança (IC) de 95%. A interação entre as variáveis será testada por regressão logística.

IV - COMENTÁRIOS DOS RELATORES

Trata-se de um estudo retrospectivo, multicêntrico (2 centros) que utilizará material biológico já coletado (material de ectocérvice e endocérvice vaginal e secreção vaginal) de 234 mulheres, por indicação clínica e já utilizado em estudos anteriores devidamente aprovados pelos CEP envolvidos. O projeto apresenta-se bem redigido, com metodologia adequada. Os critérios de inclusão e exclusão das amostras estão bem definidos. Existe uma estimativa do número de amostras a serem incluídas e a análise estatistica foi apresentada. Os aspectos éticos estão discutidos no corpo do projeto, tendo os pequisadores se comprometido à encaminhar aos médicos envolvidos informações que eventualmente possam ser importantes para os sujeitos em questão. Está sendo solicitada a dispensa do Termo de Consentimento Livre e Esclarecido. Existe orçamento detalhado e os pesquisadores tem conhecida expertise nesta área de investigação.Considero o projeto adequado a esse tipo de estudo.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar sem restrições o Protocolo de Pesquisa, dispensa do Termo do Consentimento Livre e Esclarecido, bem como todos os anexos incluídos na pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII – DATA DA REUNIÃO

Homologado na XII Reunião Ordinária do CEP/FCM, em 21 de dezembro de 2010.

Prof. Dr. Carlos Eduardo Steiner
PRESIDENTE do COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP