

RENATA CLEMENTINO GONTIJO

**INCIDÊNCIA DE LESÕES CERVICAIS SUBSEQÜENTES EM
MULHERES COM CITOLOGIA DE RASTREAMENTO NORMAL
SEGUNDO A DETECÇÃO DO PAPILOMAVÍRUS HUMANO**

Tese de Doutorado

**ORIENTADORA: Prof^a. Dr^a. SOPHIE F. MAURICETTE DERCHAIN
CO-ORIENTADORA: Prof^a. Dr^a. CECILIA MARIA ROTELI MARTINS**

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do Título de Doutor em Tocoginecologia, área de Tocoginecologia

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**Curso de Pós-Graduação em Tocoginecologia da Faculdade
de Ciências Médicas da Universidade Estadual de Campinas**

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Dedico este trabalho...

*... aos meus pais, Canavarro e Sônia, e ao meu irmão Canavarro,
que com estímulo, torcida e amor incondicional
permitiram que meus sonhos se tornassem realidade.*

*...às minhas avós Delfina e Helena (in memorian),
exemplos que quero seguir,
pela sabedoria transmitida com palavras carinhosas.*

*...à Sophie Derchain,
amiga de todas as horas,
pelo privilégio da convivência enriquecedora.*

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***“Existe somente uma idade para ser feliz,
somente uma época na vida em que é possível sonhar.
Tempo de entusiasmo e coragem em que todo desafio
é mais um convite à luta que se enfrenta
com toda disposição de tentar algo novo,
de novo, e de novo, quantas vezes for preciso...
Essa idade tão fugaz na vida chama-se PRESENTE,
e tem a duração do instante que passa...”***

Mário Quintana

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Estrutura da tese

Esta tese está sendo apresentada no formato alternativo de teses da Universidade Estadual de Campinas (Unicamp) e de acordo com o disposto em Normas, procedimentos e orientações para publicações de dissertações e teses da Faculdade de Ciências Médicas (2005).

Esta tese faz parte de um estudo denominado Latin America Screening study (LAMS). Inclui uma introdução ao tema, os objetivos do projeto de pesquisa, e um artigo original submetido para publicação no *European Journal of Obstetrics & Gynecology and Reproductive Biology*. Os métodos e os resultados obtidos estão apresentados no artigo. Em seguida, a tese apresenta a conclusão e as referências bibliográficas. No anexo foi incluído o artigo *Comparing Pap smear cytology, aided visual inspection, screening colposcopy, cervicography and HPV testing as optional screening tools in Latin America. Study design and baseline data of the LAMS study. Anticancer Research 2005, 25(5): 3469-80*, que descreve a corte transversal de todo o projeto.

A coleta de dados foi realizada no ambulatório de Patologia Cervical do Centro de Atenção Integral à Saúde da Mulher (CAISM) da Unicamp, no Centro de Saúde Santa Bárbara, da rede pública de Campinas, e no Hospital Maternidade Leonor Mendes de Barros, em São Paulo.

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Símbolos, Siglas e Abreviaturas

ASC	<i>Atypical squamous cells</i> (Células escamosas atípicas)
CAISM	Centro de Atenção Integral à Saúde da Mulher
CH II	Captura Híbrida II
CIN	<i>Cervical intraepithelial neoplasia</i>
CO	Colpocitologia oncológica
DNA	Ácido desoxirribonucléico
HC II	<i>Hybrid Capture II</i>
HPV	<i>Human Papillomavirus</i> (papilomavírus humano)
HSIL	<i>High grade squamous intraepithelial lesion</i> (Lesão escamosa intra-epitelial de alto grau)
LSIL	<i>Low grade squamous intraepithelial lesion</i> (Lesão escamosa intra-epitelial de baixo grau)
NIC	Neoplasia intra-epitelial cervical
PAP	<i>Papanicolaou</i>
PC	<i>Positive Control</i>
RLU	<i>Relative Light Unit</i>
SIL	<i>Squamous intraepithelial lesion</i> (Lesão escamosa intra-epitelial)
Unicamp	Universidade Estadual de Campinas

Resumo

Introdução: Mulheres com resultado de citologia negativa e sem infecção pelo papilomavírus humano (HPV) têm teoricamente um risco quase nulo de terem uma lesão intra-epitelial escamosa cervical de alto grau ou câncer invasor. Porém, muitas mulheres com citologia negativa e colo uterino morfolologicamente normal são infectadas pelo HPV, e o significado clínico desta infecção em relação ao risco de vir a apresentar anormalidades citológicas ou histológicas futuras ainda não está totalmente esclarecido. **Objetivo:** Investigar a incidência em 24 meses de alterações citológicas e histológicas cervicais segundo a detecção do HPV, em mulheres com citologia inicial normal, incluídas na coorte Latin American Screening study (LAMS). **Material e métodos:** Um grupo de 365 mulheres com resultado de citologia normal e resultado de Captura Híbrida II (CH II) para HPV de alto risco oncogênico positivo e negativo, foram seguidas por 24 meses em Campinas e São Paulo. Todas as mulheres responderam a um questionário referente aos fatores sociodemográficos e reprodutivos, e foram submetidas à coleta de material para citologia oncológica e CH II. As

mulheres com pelo menos um exame positivo e uma amostra aleatória de 10% de mulheres com ambos os testes negativos foram convocadas para colposcopia com biópsia, se necessário, e seguimento semestral com citologia e colposcopia. Foram comparadas as mulheres com infecção pelo HPV com aquelas não infectadas, segundo as características sociodemográficas e reprodutivas, utilizando-se o cálculo do risco relativo (RR) e a análise de regressão logística em *stepwise* com intervalo de confiança (IC) de 95%. Foram calculados também a taxa de incidência e o RR com IC de 95% de desenvolver anormalidades citológicas ou histológicas durante o seguimento. Tomou-se como padrão-ouro a colposcopia. Quando a colposcopia foi normal ou quando a biópsia apresentou cervicite, as mulheres foram consideradas como diagnóstico negativo. As mulheres cuja biópsia foi compatível com neoplasia intra-epitelial cervical (NIC) grau 1 ou mais foram consideradas como diagnóstico positivo.

Resultados: A incidência de lesões de baixo e alto graus na citologia foi maior entre as mulheres com resultado de CH II positivo, tanto aos 12 quanto aos 24 meses de seguimento. Até 12 meses de seguimento, mulheres com CH II de rastreamento positivo apresentaram um RR significativamente maior de lesões de baixo (1,4; IC 95% 1,1-1,7) e alto (1,5; IC 95% 1,4-1,7) graus na citologia. O RR para lesão de alto grau aumentou para 1,7 (IC 95% 1,5-1,9) naquelas acompanhadas por 24 meses. Em relação aos resultados histológicos, a incidência de NIC 1, 2 e 3 também foi maior entre as mulheres com resultado de CH II positivo, tanto aos 12 quanto aos 24 meses de seguimento. As mulheres com CH II positivo apresentaram um RR de 1,5 (IC 95% 1,4-1,6) para NIC 2 e 3 durante o seguimento até 12 meses e este RR aumentou para 1,7 (IC

95% 1,5-1,9) naquelas seguidas até 24 meses. **Conclusão:** O teste para detecção do HPV associado à citologia pode selecionar entre as mulheres com citologia normal aquelas com maior risco de lesão cervical subsequente.

Palavras-chave: citologia, estudo de coorte, papilomavírus humano, neoplasia intra-epitelial cervical, seguimento, colo uterino, câncer.

Summary

Introduction: Women with normal baseline cytology and non-infected by Human papillomavirus (HPV) have, in theory, no risk to develop a high-grade cervical intraepithelial lesion or cancer. However, many women with normal cytology and with morphologically normal uterine cervix are HPV infected, and, the clinical significance of this infection regarding to the risk of developing cytological or histological abnormalities in the future are not totally clear yet. **Purpose:** To investigate the incidence of cytological and histological cervical lesions in a 24 months follow-up, according to HPV detection among women with baseline normal cytology result, in a subgroup of women included in the Latin American Screening study (LAMS). **Study design:** A group of 365 women with normal Pap smear whatever the Hybrid Capture (HC) II test result were followed for 24 months at Campinas e São Paulo (Brazil). They answered a questionnaire regarding sociodemographic and reproductive factors and were submitted to a clinical exam, including Pap smear and HC II. Women with at least one positive result and a 10% random sample of women with both tests negative were referred to colposcopy and followed with cytology and colposcopy in a six-month

interval. Women with positive and negative HPV test were compared regarding sociodemographic and reproductive factors using relative risk (RR) and stepwise logistic regression analysis calculated with 95% confidence interval (CI). Also the incidence rate and RR of developing any cytological or histological abnormality during the follow-up were calculated within 95% confidence limits. Colposcopy was considered as the gold standard. When colposcopy result was normal or biopsy result was cervicitis it was considered as negative diagnosis. Women with histologic diagnosis of cervical intraepithelial neoplasia (CIN) 1 or higher were considered as positive diagnosis. **Results:** Incidence of low and high-grade cytological lesion was higher in women with positive HPV testing than in women with negative HPV testing after 12 and 24 months of follow-up. In up to 12 months of follow-up, women with baseline positive HPV test had a significantly higher proportion of low-grade (1.4; 95% CI 1.1-1.7) and high -grade (1.5; 95%CI 1.4-1.7) cytological lesion. The RR for high-grade lesion increased to 1.7 (95%CI 1.5-1.9) for those followed-up in up to 24 months. For histological outcomes, the incidence of CIN 1, 2 or 3 was also higher in women with positive HPV testing than in women with negative HPV testing after 12 and 24 months of follow-up. Women with positive HPV test had a higher RR of CIN 2 and 3 (1.5; 95%CI 1.4-1.6) during the follow-up in up to 12 months and the RR increased to 1.7 (95%CI 1.5-1.9) for those followed-up in up to 24 months. **Conclusions:** HPV test is useful in addition to cytology to select from women with normal cytology those who are at highest risk for underlying cervical lesion.

Key words: cytology, cohort study, human papillomavirus, cervical intraepithelial neoplasia, follow-up, uterin cervix, cancer.

1. Introdução

A maioria dos casos de câncer do colo uterino pode ser prevenida através do rastreamento. Em muitos países desenvolvidos, a incidência e a mortalidade por câncer cervical foram significativamente reduzidas com a implementação de programas organizados usando a citologia oncológica ou o teste de Papanicolaou (Miller et al., 2000, Syrjanen e Syrjanen, 2000; Mandelblatt et al., 2002). Entretanto, poucos países em desenvolvimento foram capazes de iniciar e sustentar programas de rastreamento baseados em citologia (Denny et al., 2000; 2002). Assim, as estimativas de incidência deste câncer para 2005 no Brasil, por exemplo, permanecem elevadas, com taxas de 22,14/100.000 mulheres (Brasil, 2004). Essa situação não é exclusiva do Brasil, e em todo o mundo, a cada ano, meio milhão de mulheres são acometidas por câncer de colo uterino e cerca de 50% delas morrem pela doença (Bosh et al., 2003). Não por acaso, 78% das novas ocorrências por ano incidem em países em desenvolvimento, evidenciando a convergência entre as condições socioeconômicas ruins e a precariedade do acesso ao diagnóstico e tratamento das lesões precursoras.

Antes do teste de Papanicolaou ser usado no início dos anos 50, o diagnóstico do câncer cervical era feito em estágios avançados, e em 60% das vezes eram inoperáveis. A citologia melhorou o diagnóstico precoce e o prognóstico destas pacientes e tornou-se o melhor método de rastreamento em relação custo-benefício, permitindo que mulheres com lesões pré-invasoras fossem tratadas a tempo. Porém, o exame não é um método infalível (Koss, 1989; 1993). Os resultados de metanálises sugerem que o rastreamento citológico tem uma enorme variação de sensibilidade para detectar lesões (Bastian et al., 1999). Como predomina claramente no exame citopatológico o trabalho manual, desde a colheita até a emissão e liberação do resultado pelo laboratório, seu desempenho está relacionado com a qualidade dos recursos humanos envolvidos (Mody et al., 2000).

Paralelamente, já está bem definida a relação causal entre papilomavírus humano (HPV) e o câncer cervical e suas lesões precursoras. Este conhecimento trouxe novas metodologias que podem contribuir para entender a evolução natural da doença e realizar seu diagnóstico precoce. Na prática clínica, muitas das infecções pelo HPV não são detectadas e regredem espontaneamente sem nenhum prejuízo para a mulher e, embora a infecção seja necessária para o desenvolvimento do câncer cervical, isoladamente não é capaz de induzir a progressão de uma célula normal para uma célula neoplásica. Mesmo em uma mulher infectada pelo vírus, o carcinoma cervical é uma consequência relativamente rara e outros fatores são necessários para influenciar esta progressão (Franco et al., 1995, Syrjanen et al., 2005).

Várias características do comportamento sexual são associadas com um risco maior para o câncer cervical, sugerindo que esta doença se comporta como uma doença sexualmente transmissível. As mais consistentes são: múltiplos parceiros sexuais, parceiros sexuais promíscuos e início da atividade sexual precoce (Gontijo et al., 2005). Além disto, o nível socioeconômico baixo, a paridade, o uso de anticoncepcional hormonal, fatores dietéticos e imunossupressão têm sido implicados como potenciais fatores de risco para o carcinoma cervical (Syrjanen e Syrjanen 1999, Atalah et al., 2001). O tabagismo é considerado como fator independente associado à presença de lesões pré-neoplásicas cervicais de alto grau (Derchain et al., 1999; Kjellberg et al., 2000, Atalah et al., 2001).

A captura de híbridos II (CH II), desenvolvida há nove anos, é um ensaio de hibridização molecular, não radioativo, de procedimento rápido e leitura confiável desenhado para detectar 18 tipos de HPV divididos em grupos de baixo (6, 11, 42, 43 e 44) e alto (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 e 68) risco oncogênico (Clavel et al., 1998; 2001; Lorincz, 1996, Lorincz et al., 2002). Baseado na premissa que a detecção do HPV em células cervicais esfoliadas poderia apresentar um desempenho diagnóstico aceitável, sendo melhor reproduzível e facilmente adaptado para a prática clínica (Saslow et al., 2002), vários estudos vêm mostrando que este teste tem o potencial para ser usado no rastreamento de lesões cervicais, em associação com outros métodos ou até isoladamente (Ratnam et al., 2000; Schiffman et al., 2000; Clavel et al., 2001). Existe uma grande correlação entre as anormalidades detectadas pelo exame citológico e a detecção do HPV, pois embora cerca de 11% das mulheres com

citologia normal apresentem HPV detectável, esta proporção atinge 73% entre aquelas com exames alterados (Wolf et al., 2003).

A CH II, entretanto, não foi aprovada para substituir a citologia, mas sim como teste adjunto ao exame de Papanicolaou, em alguns países (Meijer et al., 2000, Apgar e Brotzman, 2004, Gontijo et al., 2005). A *American Cancer Society* (ACS) - Sociedade Americana do Câncer - e o *Food and Drug Administration* (FDA) recomendam seu uso no rastreamento primário de mulheres com 30 anos ou mais, adjunto à citologia e realizado a cada três anos (Saslow et al., 2002, FDA, 2003). Deve se considerar, entretanto, que a infecção pelo HPV é transitória em 80% dos casos e apenas 20% das mulheres com um teste de CH II positivo apresentarão neoplasia intra-epitelial cervical (NIC). Embora aparentemente simples, muito pouco se conhece do impacto psicológico e emocional causado quando se relata a uma mulher que ela é portadora de uma infecção por HPV, doença sexualmente transmissível com potencial efeito carcinogênico (Nanda e Meyers, 2001, Syrjanen et al., 2005).

O rastreamento nas mulheres com idade acima de 30 anos tende a melhorar a especificidade do teste consideravelmente, pois as infecções virais nesta faixa etária são menos comuns e a probabilidade de serem de natureza transitória é menor que em mulheres mais jovens (Ratnam et al., 2000, Arbyn et al., 2004).

O custo dos testes de HPV, entretanto, permanece como um impedimento maior para sua aceitação em programas de prevenção de câncer (Goldie et al.,

2001). O cálculo do custo/efetividade vai variar muito de uma população para outra e está relacionado não só ao custo do exame mas à infra-estrutura disponível, o intervalo já preconizado entre os exames e o controle de qualidade vigente por cada legislação. Em países com alto padrão de vida, como o Reino Unido, Holanda, França e Itália, o rastreamento com teste de HPV tem o potencial de aumentar os benefícios dos programas de saúde a um custo razoável. Este custo foi calculado em torno de US\$13.000 por ano/vida salva, o que é provavelmente inaceitável para a maioria dos países em desenvolvimento, especialmente em se tratando de programas financiados pelo governo. O custo varia significativamente de um país para outro, e não foi calculado ainda no Brasil, no qual o sistema público de saúde reembolsa aos hospitais e laboratórios cerca de US\$2 por citologia realizada. Por outro lado, a CH II custa cerca de US\$45 o exame, o que restringe seu uso para o setor privado (Kim, Wright e Goldie, 2005, Syrjanen et al., 2005).

Apesar destas questões, existe um interesse crescente em utilizar testes de HPV em associação com a citologia, tanto em programas de rastreamento como no seguimento de mulheres com alterações citológicas, para determinar a combinação com maior desempenho na detecção de lesões. Com o objetivo de obter dados em uma amostra de mulheres da América Latina, foi desenvolvida uma linha de pesquisa prospectiva visando avaliar a “Melhoria da qualidade dos programas de controle de câncer do colo do útero na América Latina, comparando a citologia oncológica, inspeção visual, cervicografia e teste para detecção do HPV como otimizador de rastreamento no Brasil e Argentina” (Latin America Screening

study – LAMS) (Anexo 1), esperando-se que a aplicação de um instrumento diagnóstico mais simples e o custo efetivo possam resultar em melhoria da detecção de lesões neoplásicas e pré-neoplásicas cervicais em nosso meio.

Resultados parciais deste estudo, descritos previamente, demonstraram que entre mais de 2000 mulheres rastreadas com citologia, inspeção visual com ácido acético e CH II em Campinas e São Paulo, quase um terço apresentaram pelo menos um teste alterado. A sensibilidade da CH II (65%) para detectar lesões prevalentes foi ligeiramente superior à da citologia (54%). A baixa especificidade da CH II (74%) resultou em maior número de resultados falso-positivos, o que pode ser explicado pelo fato de a população estudada ser jovem, na qual provavelmente a maioria das infecções é transitória e regride espontaneamente, sem evoluir para lesão (Gontijo et al., 2004, Gontijo et al., 2005). Por outro lado, outros estudos que avaliaram a CH II no rastreamento referem sensibilidade bem maior que a da citologia, próxima de 100% para detecção de lesão de alto grau, mantendo a especificidade significativamente menor (Schiffman et al., 2000, Clavel et al., 2001).

Mais importante talvez do que o desempenho dos testes de detecção viral, é a estimativa de risco que uma mulher com citologia normal possa vir a desenvolver ou apresentar NIC ou câncer cervical durante o seguimento (Arora et al., 2005). Alguns estudos têm demonstrado relação entre a detecção do HPV e o risco aumentado de alteração cito/histológica futura em mulheres com citologia inicialmente normal (Bory et al., 2002, Castle et al., 2003, Dalstein et al., 2003, 2004). Mulheres com alta carga de HPV 16 apresentaram duas a 68

vezes mais risco de evoluir para NIC 3 quando comparadas com mulheres nas quais o HPV 16 não foi detectado (Josefsson et al., 2000, Ylitalo et al., 2000, Van Duin et al., 2002). Estes dados confirmam que a infecção pelo HPV precede o desenvolvimento de lesão cervical, e indicam que mulheres com citologias normais e infectadas pelo HPV devem ser acompanhadas com mais frequência que aquelas com o teste negativo.

Vários autores inferem que mulheres com ambos os exames negativos poderiam ser submetidas a novo rastreamento em intervalos maiores, pela menor probabilidade de desenvolverem alguma lesão (Bory et al., 2002, Dalstein et al., 2003, Cuzick et al., 2003, Dalstein et al., 2004). Se suficientemente específica, esta abordagem permitiria a um grande grupo de mulheres com citologia normal e CH II negativa evitar repetir testes freqüentes e desnecessários, sendo possível focalizar os programas de rastreamento nas mulheres com maior risco. Entretanto, ainda faltam estudos clínicos prospectivos randomizados populacionais que possam validar esta teoria (Sherman et al., 2003).

Neste contexto, este estudo pretende verificar a incidência e o risco relativo de lesão cervical subsequente em um subgrupo de mulheres com citologia inicial normal, segundo o resultado do teste de HPV de rastreamento, durante um seguimento cito-colposcópico de 24 meses.

2. Objetivos

2.1. Objetivo geral

Avaliar a incidência de lesão cervical subsequente em mulheres com citologia de rastreamento normal segundo a detecção do HPV.

2.2. Objetivos específicos

- Avaliar os fatores sociodemográficos e reprodutivos associados ao risco de infecção pelo HPV.
- Avaliar a incidência e o risco relativo de alterações citológicas segundo o resultado do teste de HPV inicial no seguimento de 12 e 24 meses.
- Avaliar a incidência e o risco relativo de alterações histológicas segundo o resultado do teste de HPV inicial no seguimento de 12 e 24 meses.

3. Publicação

Artigo 1

Gontijo RC, Derchain SF, Roteli-Martins CM, Bastos JB, Sarian LO, Morais SS, Longatto-Filho A, Syrjänen K. Incidence of cervical lesions in women with baseline normal cytology according to human papillomavirus detection. Submetido para o *European Journal of Obstetrics and Gynecology and Reproductive Biology*.

3.1. Artigo 1

Incidence of cervical lesions in women with baseline normal cytology according to human papillomavirus detection

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Condensation

Usefulness of HPV test in addition to cytology to select from women with normal cytology those who are at highest risk for underlying cervical lesion

Incidence of cervical lesions in women with baseline normal cytology according to human papillomavirus detection

Objective: To investigate the incidence of cytological and histological cervical lesions in a 24 months follow-up, according to HPV detection among women with baseline normal cytology, in a subgroup of women included in the Latin American Screening study (LAMS). **Study design:** A group of 365 women with normal Pap smear with negative and positive high-risk Hybrid Capture II test were prospectively followed in up to 24 months at Campinas e São Paulo (Brazil). The relative risk (RR) of developing any cytological or histological abnormality during the follow-up was calculated with 95% confidence interval (CI). **Results:** Until 12 months of follow-up, women with baseline positive HPV test had a significantly higher proportion of low-grade (1.4; 95%CI 1.1-1.7) and high-grade (1.5; 95%CI 1.4-1.7) cytological lesion. The RR for high-grade lesion increased to 1.7 (95%CI 1.5-1.9) for those followed in up to 24 months. For histological outcomes, women with positive HPV test had a higher proportion of cervical intraepithelial neoplasia 2 and 3 (1.5; 95%CI 1.4-1.6) during the follow-up in up to 12 months and the RR increased to 1.7 (95%CI 1.5-1.9) for those followed in up to 24 months. **Conclusions:** HPV test is useful in addition to cytology to select from women with normal cytology those who are at highest risk for underlying cervical lesion.

Key words: negative cytology, hybrid capture II, follow-up, CIN

Introduction

Several epidemiological studies in the last decade have established a strong relation between high-risk types of human papillomavirus (HR-HPV) and the development of cervical cancer and precancerous lesions [1,2]. Indeed, it has been reported that 99.7% of all cervical squamous cells carcinomas contain HR-HPV [1].

Insight into this relation between HR-HPVs and the natural history of squamous intraepithelial lesions (SIL), it has been proposed that testing for the presence of these viruses in cervical scrapes as an adjunct of cervical cytology could be of great value in a variety of clinical settings [2]. The commercial available Hybrid Capture (HC) II, is a non radioactive, rapid, reproducible and sensitive assay, designed to detect 18 HPV types divided into high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 e 68) and low-risk (types 6, 11, 42, 43 e 44) groups [3,4,5].

HC II test is being proposed as a general population screening test in conjunction with the Papanicolaou (Pap) test for women 30 years of age and older, as reassurance of the absence of high-grade SIL (HSIL) or invasive carcinoma [6,7]. However, most HPV DNA positive women are cytologically negative, considering that most HPV infections are transient and not associated with detectable cytological abnormality. Many authors consider that a positive HPV testing selects a population of highest risk of developing a HSIL, while a concurrent negative Pap smear and negative HC II test have a substantially decreased risk of cervical lesion, providing strong reassurance that incident disease will be absent [6,8,9,10,11]. Consequently, the knowledge about clinical significance of an HPV-positive, cytologic-negative result with regard to prediction of subsequent cervical lesions is still needed.

Thus, we carried out a prospective study to investigate the role of HR-HPV detected at enrollment visit in the development of SIL in women who had no previous diagnosis of cytological abnormalities, during a 24-month follow-up.

Material and methods

Study design

The subjects of this study represent a group of women derived from a major cohort study (LAMS- Latin America Screening study), run between March 2002 and May 2005 in three outpatient clinics in Brazil: Hospital Leonor Mendes de Barros, Sao Paulo; Campinas State University (UNICAMP) and Santa Barbara Public Health Center, Campinas. A description of the study design and methods has been detailed elsewhere [12]. Women were considered eligible if they met all of the following requirements: a) had an intact uterus (i.e. no previous surgical procedure of the cervix corpus); b) had no history of an abnormal Pap test in the past year; c) were not under treatment for genital condyloma (external or in the cervix); d) did not have any confirmed or clinically suspect immunosuppression (HIV, corticosteroids, chemotherapy, other chronic diseases that might compromise the immune system). They answered a questionnaire regarding reproductive and socio-demographic factors followed by a complete physical examination with Pap smear and HC II performed. Women with at least one positive result and a 10% random sample of women with both tests negative were referred to colposcopic examination. Whenever colposcopy revealed an abnormal pattern, a directed punch biopsy was taken. Women had their second visit scheduled after 45 days, to become informed about their exam/biopsy results and to be allotted to either the

treatment or the follow-up group. The local Ethics Committee approved the study protocol and all patients signed the informed consent.

Patient's selection

From a cohort of 2597 women with HC II and Pap smear enrolled in the study, 284 women with abnormal Pap result (atypical squamous cells (ASC), atypical glandular cells (AGC), low grade SIL, high grade SIL and suggestive of invasive cancer) and/or abnormal histology result (cervical intraepithelial neoplasia -CIN)- grade 1,2 or 3) at the enrollment visit were excluded. Within this cohort, we identified a subgroup of 2313 women who were cytologically negative at baseline and tested either positive or negative for oncogenic HPV types.

Patients with positive HPV test (336 women) were referred to follow-up consultations, including Pap smear collection and colposcopy at 6-month interval up to 24 months. From these, 211 returned. Among the 1979 women with negative HPV test, a 10% random sample (197 women) was also invited for the same follow-up consultations and 154 women returned. For the purposes of this analysis, we restricted participation to the 365 women who had returned to at least one follow-up visit. Figure 1 shows the different exclusions and follow-up outcomes for the entire cohort.

Follow-up Visits

Follow-up consultations comprised clinical history and gynecological examination with Pap smear and colposcopy in a six-month interval. Targeted biopsies of the cervix were taken when abnormal epithelium was found. Accordingly, the same

routine was adopted at following visits. Altogether 287 women completed the 6 and 12-month follow-up visit and 234 completed the 18 and 24-month follow-up visit.

Hybrid Capture II

The specimens for HC II were tested at the enrollment visit with probe B (HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Samples were classified as positive for HR-HPV if the relative light unit (RLU) reading obtained from the luminometer was equal or greater than the mean of the three positive control (PC) values supplied by the HC II kit (≥ 1 pg/ml RLU/PC). These RLU/PC ratios also provide a semi-quantitative estimate of the amount of HPV DNA in the specimens, i.e., the viral load in the sample. The storage of the specimens and all reagents as well as conduction of the tests took place at the Medical School Hospital Laboratory, following the manufacturer's instructions (*Digene Diagnostics Inc., USA*).

Cervical cytology

Screening cervical cytology was tested in two modes: conventional and liquid-based cytology (LBC) techniques. Conventional Pap smear was taken by Campinas centre in 285 women, following conventional procedures of smear taking, fixation and staining. The system of LBC was tested in 80 women at Sao Paulo centre using DNA-Cytoliq® (*Digene Brazil, Sao Paulo Brazil*). The samples were collected using the brush of DNA-Cytoliq system and immersed in the Universal Collecting Medium vials. The sample processing followed the manufacturer's instructions [13]. At the follow-up visits, only conventional cytology was performed. Slides were fixed in absolute ethanol and stained by the modified Papanicolaou method. Final cytological diagnoses were

rendered using the Bethesda System [14] and were classified as normal/inflammatory, ASC, LSIL or HSIL. For statistical purposes, normal/inflammatory exams were grouped as negative and ASC, LSIL or HSIL as positive.

Colposcopy

Colposcopy was performed for all women who comprised follow-up visits. Experienced and certified colposcopists performed all examinations. The classification was according to the terminology of the International Federation of Cervical Pathology and Colposcopy (IFCPC) [15].

Histology

Biopsy specimens were fixed in 10% phosphate buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE). Pathological diagnoses were rendered according to the World Health Organization [16] criteria's and classified as negative, CIN 1, CIN 2 or CIN 3. For statistical analysis, results of cervical biopsies were grouped as negative when normal/cervicitis was present and positive when showing CIN 1, CIN 2 or CIN 3.

Statistical analysis

We calculate the association between HPV-positive test and some reproductive and sociodemographic factors using relative risk (RR) with stepwise logistic regression analysis. We also calculated the incidence rate and RR of developing any cytological or histological abnormality during the follow-up among women with HPV-positive or HPV-negative test at enrollment. Colposcopy was considered as the gold standard. When

colposcopy result was normal or biopsy result was cervicitis it was considered as negative diagnosis. Women with histological diagnosis of CIN 1 or higher were considered as positive diagnosis. All calculations were performed with EpiInfo V3.0 (Centers for Disease Control, Atlanta, USA) within 95% confidence limits.

Results

The women in the study group ranged between 15 and 65 years of age with the mean age of 32 years (data not shown). In a crude analysis, single women were more likely to have an HPV infection (RR=1.4 95%CI 1.2-1.7). Also women who had the first intercourse with less than 18 years old (RR=1.4 95%CI 1.1-1.6), had more than two lifetime sexual partners (RR=1.6 95%CI 1.3-1.9) and more than two partners in the last 12 months (RR=1.5 95%CI 1.2-1.8) were more likely to have a positive HPV test result. Age, parity and modes of contraception (hormonal, condom, intrauterine device, tubal sterilization and others) were not associated with HPV infection. Partner previous sexual transmitted disease (RR=1.5 95%CI 1.2-1.8) showed increased risk of HPV infection. This risk was not associated with women who never smoke, who were former smoker or who smoked in the past. After the logistic regression analysis, only women who had more than two lifetime sexual partners (RR=1.5 95%CI 1.0-2.2) remained significantly associated with a positive HPV testing (**Table 1**).

We examined the incidence rate and the relative increase in risk of developing any cytological abnormality during a 24-month follow-up among those women who were cytologically negative and either HPV-positive or HPV-negative at enrollment. In up to 12 months of follow-up, the incidences of LSIL and HSIL were 4.6% and 1.0%

respectively for women with positive HPV test, compared to 1.1% and 0% for those with negative HPV test result. In up to 24 months, the incidence of LSIL cytology decreased to 2.1% and the incidence of HSIL cytology increased to 2.1% for women with positive HPV test. Women with baseline positive HPV test had a significantly higher RR of LSIL (1.4; 95%CI 1.1-1.7) and HSIL (1.5; 95%CI 1.4-1.7). There was a trend for an increase in RRs of HSIL over time, with point estimates increasing from 1.5 (95%CI 1.4-1.7) in up to 12 months follow-up to 1.7 (95%CI 1.5-1.9) for those followed-up in up to 24 months. During the 24 months follow-up, there was no HSIL observed among those with negative HPV test. Nevertheless, a small proportion of LSIL and ASC results were observed (**Table 2**).

We repeated the analysis separately for histological outcomes. In up to 12 months of follow-up, the incidences of CIN 1 and CIN 2/3 were 6.2% and 2.6% respectively for women with positive HPV test, compared to 3.2% and 0% for those with negative HPV test result. In up to 24 months, the incidences of CIN 1 and CIN 2/3 decreased to 2.1% and 0.7% respectively for women with positive HPV test. Women with baseline normal cytology and positive HPV test had a significantly higher RR of CIN 2 and 3 (1.5; 95%CI 1.4-1.6) during the follow-up in up to 12 months. At the follow-up in up to 24 months, the RR of CIN 1 (1.7; 95%CI 1.5-1.8) was also remarkably higher. There was observed a similar trend of increasing RR of CIN 3 over time, from 1.5 (95%CI 1.4-1.6) for women followed-up in up to 12 months to 1.7 (95%CI 1.5-1.8) for those followed-up in up to 24 months. Similarly to cytological analysis, in HPV-negative women followed-up in up to 12 months, two cases of CIN 1 were observed and there was no CIN 3 diagnosed over time (**Table 3**).

Discussion

To date, the detection of cervical cancer and its precursors by Pap test is widely recognized as the most effective method for preventing carcinoma of the cervix [2,10,17]. However, cytological screening suffers from limited reproducibility and high rate of false negative and false positive results [18,19]. This limitation is further compounded by a substantial number of Pap smears being reported as negative that may represent an unrecognized precursor to atypia in many cases [20].

To overcome this problem, with the knowledge about the role of HPV in natural history of cervical lesions, it has been suggested that testing for HPV as an adjunct of cytology could dramatically improve the detection of cervical disease [2,11]. Furthermore, the fact the HPV test has high sensitivity and low specificity and Pap smear low sensitivity and high specificity could be used to support each other.

In the present study, the overall prevalence of HR-HPV in women with negative Pap smear was 14%, whereas others have observed HPV prevalence ranging from 10% [21] to 21% [22]. Risk for HPV infection was associated with single women, age at first intercourse, number of lifetime sexual partners and partner with previous sexual transmitted disease. It suggests that sexual behavior increases the probability of contact with an infected partner and reflects the important role of sexually transmitted agents in the etiology of cervical lesions [21,23,24,25]. However, hormonal contraceptive use, multiparity and smoking, factors that are thought to influence the likelihood of HPV infection [26,27] did not show this association in the population studied. After logistic regression analysis, only the number of lifetime sexual partners remained associated with having an HPV infection.

The incidence rates for HSIL cytology and CIN 2/3 in women with positive HPV testing were respectively 1.0% and 2.6% in women followed-up in up to 12 months. For those followed-up in up to 24 months, the incidence rate for HSIL cytology increased to 2.1% and the incidence rate for CIN 2/3 decreased to 0.7%.

This study also indicates that over the entire duration of follow-up, women with baseline negative Pap smear and positive HPV test are more likely to develop HSIL cytology and CIN 3 histology result as those with both tests negative. This is consonant with earlier articles that provide confirmation that HPV infection precedes the development of CIN [8,9,11,21,27,28]. In a study carried out by Rozendaal et al [8], among 1622 women followed for a mean of 40 months, HPV detection was associated with 116-fold increased risk for the development of CIN 3 in contrast to women without HR-HPV. Evaluating 496 women attending a family planning clinic, Moscicki et al [28] reported that approximately 20% of participants with an HPV infection were subsequently found to have a Pap test interpreted as LSIL during a follow-up of 50 months. Bory et al [22] evaluated 3091 women with normal smears and found a relative risk of 96.7 of incident HSIL when HPV was detected at enrollment.

For instance, Kjaer et al [9] followed 10177 cytologically normal women and observed that HPV status at enrollment predicted future development of high-grade lesions. Castle et al [6] also described that about 17% of 2511 women attending screening clinic with a negative Pap test and a positive HPV DNA test will develop an abnormal Pap within approximately five years. Relying merely on simple HPV detection as a biomarker of increased risk of subsequent cervical lesion suffers from some practical limitations. We have observed a small proportion of LSIL and ASC cytology in HPV-negative women during the follow-up. It could be explained for three reasons: 1)

fluctuations on viral load below the detection threshold of screening tests leading to misclassification of some infected women as false negative [29], 2) HPV infection could be due to low-risk types, which were not evaluated in this study, 3) it is likely that some subsequent abnormal Pap tests called ASC, and even a few LSIL, were not due to any HPV infection, particularly in older women. They were a consequence of age-related atrophic changes in the cervical epithelia that mimic equivocal cytologic changes [6].

However, regarding histological outcome, as well as Dalstein et al [30] and Sherman et al [11] described, we found no high-grade SIL among women who tested negative for HPV during the entire follow-up. The low risk of lesion among women with both negative tests reflects mainly the high sensitivity and negative predictive value of HPV testing. In consequence, as previous suggested several studies, in such conditions, the use of HPV testing would allow the screening interval to be safely lengthened [8,10,22,30,31]. In the other hand, a single positive test for oncogenic HPV predicts increased risk for disease [11] and women with positive HPV test and a negative Pap test should be targeted for more frequent surveillance.

A limitation of our trial is that only the screening HC II test result was used for predicting the RR of disease during the follow-up. Bory et al. [22] have shown that an HPV test remaining positive at two controls is significantly associated with an increased risk of incident HSIL. Moreover, a second negative HR-HPV test one or two years after the initial test can reach a negative predictive value of 100% [32], reassuring that women with double negative tests really represent a low risk population of incident disease. Taking this into account, this ongoing study has extended follow-up and we have been collecting another samples for HPV after one, two and three years of follow-up. This approach will provide more adequate analysis of the duration of the risk after a positive

HPV test, which might lead to better understanding of the natural history of squamous intraepithelial lesions.

Results from this study and others [6,10,11,21,22,28] highlight the central etiologic role of HPV in cervical lesions by establishing that viral infection precedes the development of disease. It is, therefore, suggested that while Pap test should continue to be the routine screening method for detection of cervical lesions, HPV testing can be utilized as an adjunct to cytology for effective screening, especially for those women with negative Pap smear [16, 21].

In summary, our study using HC II assay confirms the usefulness of this test in addition to cytology to select from a population of women with normal cytology those who are at highest risk for underlying cervical lesion.

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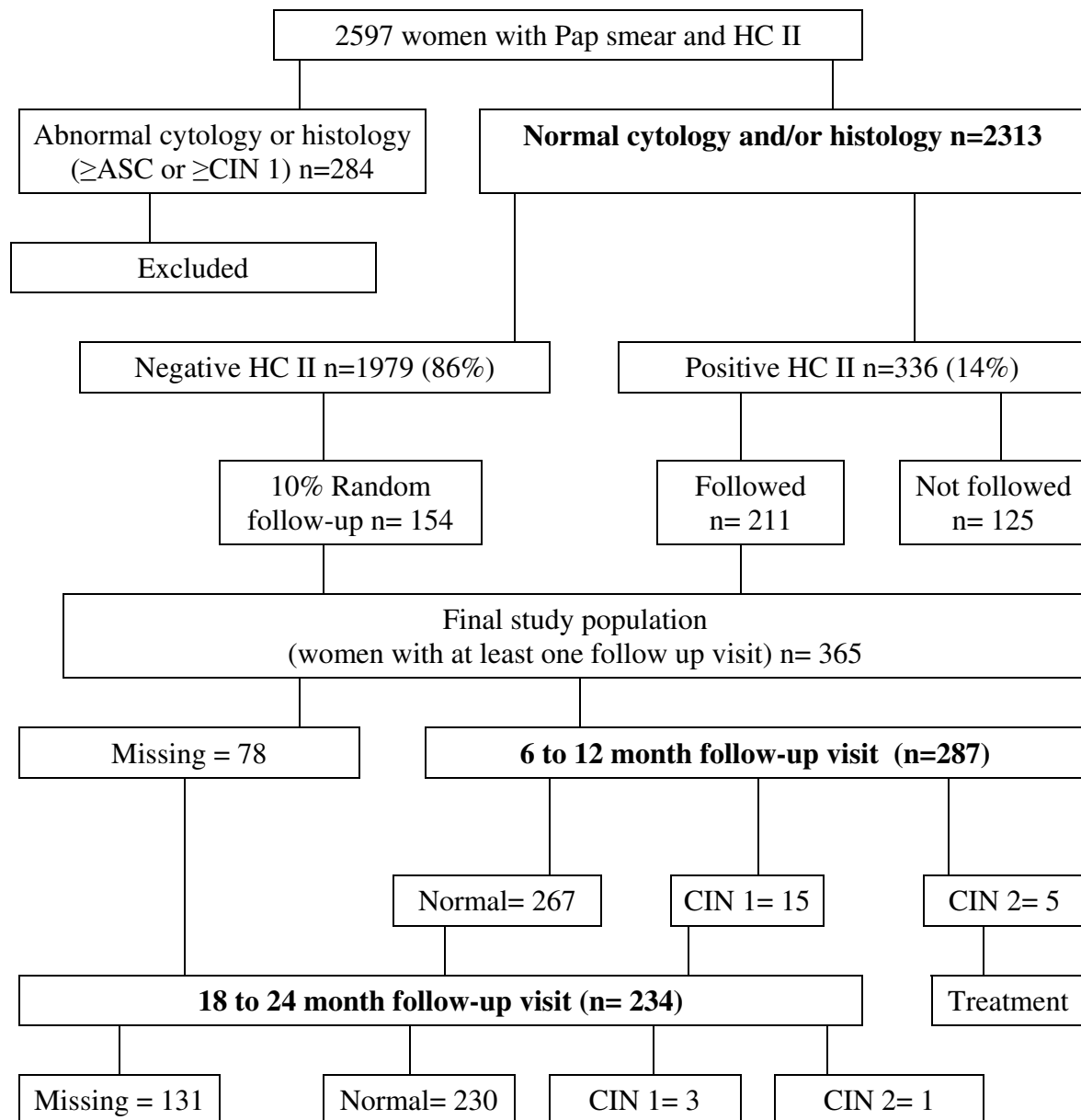


Figure 1: Schematic overview of overall study design.

Table 1: Epidemiological risk factors and their association with the risk of HPV infection in women with negative Pap smear

Variables	HPV		Crude RR (95% CI)	Adjusted RR (95% CI)
	HPV positive/total	(%pos)		
Age				
< 30 years	100/157	(63.6)	Ref	
≥30 years	111/207	(53.6)	0.8 (0.7-1.0)	NS
Marital status				
Living with partner	122/240	(50.8)	Ref	
Single	88/124	(70.9)	1.4 (1.2-1.7)	NS
First intercourse				
≥18 years	84/172	(48.8)	Ref	
<18 years	127/193	(65.8)	1.4 (1.1-1.6)	NS
Lifetime partners				
<2	60/140	(42.8)	Ref	
≥2	151/225	(67.1)	1.6 (1.3-1.9)	1.5 (1.0-2.2)
Number of partners in last 12m				
<2	191/341	(56)	Ref	
≥2	20/24	(83.3)	1.5 (1.2-1.8)	NS
Parity				
0	88/161	(54.6)	Ref	
1-2	82/131	(62.5)	1.2 (0.9-1.4)	NS
≥2	41/72	(56.9)	1.0 (0.8-1.3)	NS
Contraceptive method				
No contraception	37/59	(62.7)	Ref	
Hormonal	77/118	(65.2)	1.0 (0.8-1.3)	NS
Condom	34/52	(65.3)	1.0 (0.8-1.4)	NS
IUD	12/31	(38.7)	0.6 (0.4-1.0)	NS
Tubal sterilization	24/50	(48.0)	0.8 (0.5-1.1)	NS
Others	27/55	(49.0)	0.8 (0.6-1.1)	NS
Partner STD				
No	172/313	54.9	Ref	
Yes	28/35	80	1.5 (1.2-1.8)	NS
Smoking habit				
Never	138/231	51	Ref	
Yes < 1 pack/day	29/55	52.7	0.9 (0.7-1.2)	NS
Yes ≥ 1 pack/day	18/27	66.6	1.1 (0.8-1.5)	NS
Past < 1 pack/day	22/41	50	0.9 (0.7-1.2)	NS
Past ≥ 1 pack/day	4/11	36.3	0.6 (0.3-1.3)	NS

CI: Confidence interval, IUD: Intra uterine device, STD: Sexual transmitted disease

Table 2: Risk of cytological abnormalities during follow-up associated with result of HPV at enrollment

Cytology result	Total	HPV negative		HPV positive		RR	(95 % CI)
		N	Incidence rate	N	Incidence rate		
First visit							
(up to 12 months)							
Normal	246	84	90.3%	162	83.5%	Ref	
ASC	29	8	8.6%	21	10.1%	1.1	(0.9 – 1.4)
LSIL	10	1	1.1%	9	3.5%	1.4	(1.1 – 1.7)
HSIL	2	0	0%	2	0.7%	1.5	(1.4 – 1.7)
Total		93		194			
Missing		61		17			
Second visit							
(up to 24 months)							
Normal	206	82	90.1%	124	86.7%	Ref	
ASC	20	7	7.7%	13	9.1%	1.1	(0.8 – 1.5)
LSIL	5	2	2.2%	3	2.1%	1.0	(0.5 – 2.1)
HSIL	3	0	0%	3	2.1%	1.7	(1.5 – 1.9)
Total		91		143			
Missing		63		68			

RLU relative light unit, LSIL low-grade squamous intraepithelial lesion, HSIL high-grade squamous intraepithelial lesion, ASC atypical squamous cells

Table 3: Risk of histological abnormalities during follow-up associated with result of HPV at enrollment

Biopsy result	Total	HPV negative		HPV positive		RR	(95 % CI)
		N	Incidence rate	N	Incidence rate		
First visit							
(up to 12 months)							
Normal (Biopsy or colposcopy)	267	90	96.8%	177	91.2%	ref	
CIN 1	15	3	3.2%	12	6.2%	1.2	(0.9 – 1.6)
CIN 2/3	5	0	0%	5	2.6%	1.5	(1.4 – 1.6)
Total		93		194			
Missing		61		17			
Second visit							
(up to 24 months)							
Normal (Biopsy or colposcopy)	230	91	100%	139	97.2%	ref	
CIN 1	3	0	0%	3	2.1%	1.7	(1.5 – 1.9)
CIN 2/3	1	0	0%	1	0.7%	1.7	(1.5 – 1.9)
Total		91		143			
Missing		63		68			

CIN cervical intrepithelial neoplasia

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4. Conclusões

- Em análise univariada, ser solteira, iniciar atividade sexual com menos de 18 anos, ter mais do que dois parceiros sexuais durante a vida, mais que dois parceiros nos últimos 12 meses e história de doença sexualmente transmissível prévia no parceiro estiveram significativamente associados à infecção pelo HPV. A idade, paridade, diferentes métodos contraceptivos e o tabagismo não estiveram relacionados com a infecção pelo HPV. Após regressão logística em *stepwise*, apenas o fato de ter mais que dois parceiros sexuais durante a vida manteve-se significativamente associado com a infecção pelo HPV.
- A incidência de lesões citológicas de baixo e alto grau foi maior nas mulheres com teste de HPV positivo, tanto aos 12 meses quanto aos 24 meses. O risco relativo de lesões citológicas de baixo e alto grau foi maior até os 12 meses de seguimento nas mulheres com HPV detectável no rastreamento, e o risco relativo para lesão de alto grau aumentou no seguimento de até 24 meses. Não foi detectado um único caso de lesão de

alto grau durante os 24 meses de seguimento em mulheres com CH II negativa, embora tenha sido observada uma pequena proporção de mulheres com lesão de baixo grau e atipias de células escamosas.

- Em relação ao diagnóstico histológico, a incidência de NIC 1, 2 ou 3 foi maior nas mulheres com teste de HPV positivo, tanto aos 12 meses quanto aos 24 meses. Mulheres com o teste de HPV positivo apresentaram risco relativo significativamente maior de NIC 2 e 3 nos primeiros 12 e 24 meses de seguimento, e após 24 meses, o risco de desenvolver NIC 1 também foi significativamente maior nestas mulheres. Não foi observado um único caso de NIC 3 em mulheres com CH II de rastreamento negativa.

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7. Anexos

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