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Análise de carga viral e estado físico do Papilomavírus Humano (HPV) e perfil de citocinas em mulheres com lesão intraepitelial cervical e carcinoma cervical invasor.

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Patologia

Orientadora: Profa. Dra. Márcia Guimarães da Silva

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Palavras-chave: Carga Viral; Citocinas; Estado físico; HPV; Progressão lesão cervical.

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1. Revisão da Literatura

Os Papilomavírus Humano (HPVs) são vírus DNA, pequenos, pertencentes ao gênero *Papillomavirus* e a família *Papillomaviridiae*. Suas partículas medem aproximadamente 55nm de diâmetro, tem forma icosaédrica e não são envelopados. O DNA viral é associado com proteínas *histone-like*, possuindo aproximadamente 8000 pares de bases e é encapsulado por 72 capsômeros[1]. Foram descritos mais de 120 tipos que infectam o homem e representam diferentes repercussões clínicas[1], destes, aproximadamente 40 infectam especificamente o trato anogenital[2,3]. Os HPVs apresentam tropismo para infectar a camada basal de epitélios, através de micro lesões, induzindo lesões proliferativas[2].

A organização molecular do genoma do HPV é dividida em três domínios: genes precoces E1, E2, E4, E5, E6 e E7; genes tardios, L1 e L2 que codificam as proteínas do capsídeo viral, e a região LCR, controladora da expressão gênica e da replicação do DNA; sendo que as regiões E6, E7 e L1 se apresentam com maior similaridade entre os diferentes genótipos[4]. Os genes da região precoce E1 e E2 estão envolvidos na replicação e no controle da transcrição do DNA viral; E4 na maturação e liberação das partículas virais para o meio extracelular[5] e E5, E6 e E7 na imortalização e transformação celular[6], codificando proteínas relacionadas a funções de estimulação do crescimento e evasão do sistema imune[7]. O gene E5 aumenta a sinalização iniciada pelo receptor do fator de crescimento epidermal (EGF), estimulando a proliferação celular e a replicação viral[8,9], sendo expresso somente nos estágios iniciais da infecção pelo HPV[7]. Os genes E6 e E7 são expressos na

forma de um único RNA mensageiro (RNAm) policistrônico transcrito da região promotora E6/E7.

As oncoproteínas E6 e E7 ao interferirem diretamente no ciclo celular, induzem uma fase S alterada e consequente alteração da duplicação do DNA celular. A proteína E6, codificada por HPV de alto risco, é capaz de se ligar a proteína celular E6AP, degradando a proteína supressora de tumor p53 através da via da ubiquitinação[10-12]. A associação de E7 com a proteína pRb está bem caracterizada na literatura[13-15]. A pRb é reguladora negativa do ciclo celular que impede a entrada na fase S e está associada a família E2F de fatores de transcrição. A ligação E7/pRb libera o fator de transcrição E2F do complexo pRB-E2F levando a expressão de proteínas necessárias para a replicação do DNA viral[13-15]. Dessa maneira, E6 e E7 contribuem para a imortalização das células infectadas pelo vírus[16].

Os métodos de diagnósticos morfológicos permitem a identificação de lesões HPV induzidas. Resumidamente, são disponíveis os exames clínicos (inspeção visual com adição de lugol ou ácido acético), colposcopia, citologia oncótica e histopatologia. Já a identificação da infecção por HPV propriamente dita é realizada por métodos de biologia molecular, tais como captura híbrida, hibridização *in situ*, análise de restrição de fragmentos polimórficos (RFLP), reação em cadeia da polimerase (PCR) (*Nested, multiplex*, tempo real), microarray e uso de kits comerciais (Amplicor e Linear Array)[17-21]. Com a melhoria da qualidade das metodologias moleculares utilizadas para a identificação do DNA viral, a prevalência do HPV passou a ser detectado em 70-90% das LIEAG, 20-50% em LIEBG, 50% das atipias de significado indeterminado em células escamosas (ASCUS) e em atipias de células

glandulares (AGUS), 70-90% em câncer anal, 50 a 90% em câncer de pênis e 33-72% em câncer de cavidade oral e orofaringe[22-24].

Infecção pelo HPV e Genotipagem

O primeiro passo da infecção viral ocorre com a adesão da partícula viral na célula, interagindo com receptores, os quais permitem a entrada do mesmo, através do reconhecimento de proteínas produzidas pelo próprio vírus. Essa infecção está relacionada com o grau de diferenciação do epitélio e ocorre nas células epiteliais da camada basal, através de micro lesões. Após a entrada da partícula viral, há formação de vesículas e essas levam os vírus até o núcleo da célula. Os poros da membrana nuclear podem servir de porta de entrada para tais agentes, que ao entrarem no núcleo, fundem o seu genoma com o da célula hospedeira, utilizando a alta capacidade destas células de se reproduzirem, fazendo com que este produza RNAm para proteínas virais[1]. Embora o vírus possa infectar apenas as células escamosas imaturas, a replicação de HPV ocorre nas células escamosas em maturação resultando em um efeito citopático, “atipia coilocítica” que consiste em atipia nuclear e um halo citoplasmático perinuclear[25]. Nas camadas mais diferenciadas do epitélio, o DNA viral é replicado, as proteínas do capsídeo são formadas e a progênie viral é liberada junto com a descamação das células epiteliais[5].

Os HPVs genitais são classificados de acordo com seu potencial na indução do câncer em genótipos de alto risco oncogênico (16,18,31,33,35,39,45,51,52,58 e 69) e genótipos de baixo risco oncogênico (6,11,40,42,43,54,61)[2,14,26,27]. Os HPVs de baixo risco estão predominantemente associados aos condilomas genitais benignos, sendo os

mais comuns os genótipos 6 e 11. Os HPvs de alto risco, principalmente os tipos 16 e 18, estão associados ao desenvolvimento das lesões precursoras e do câncer do colo do útero, que são geralmente causados pela integração do genoma viral ao DNA do hospedeiro[14,28].

Lesões HPV induzidas

As lesões intraepiteliais cervicais abrangem o espectro de anormalidades epiteliais escamosas não invasivas associadas ao HPV, que vão desde as alterações celulares associadas à infecção transitória pelo HPV, até as alterações celulares anormais que, segundo o sistema Bethesda[29] são divididas em lesão intraepitelial escamosa de baixo grau (LIEBG) e alto grau (LIEAG). Nas LIEBG, evidenciam se células superficiais e/ou intermediárias com núcleos aumentados de até 50% do tamanho da célula, com perfil irregular, disceratose, cromatina geralmente em grumos grosseiros, binucleação ou multinucleação, presença de coilócitos, com células escamosas atípicas imaturas confinadas ao terço inferior do epitélio estando associadas à infecção produtiva por HPV. Já nas LIEAG, observam-se alterações mais intensas como células menos maduras em relação ao tamanho das células parabasais, dispostas em agregados do tipo sincício ou isoladas e grupos celulares coesos em fileira, núcleo aumentado em até dois terços do tamanho da célula, hipercromático, com cromatina granulosa, multinucleação, contorno irregular da membrana nuclear e aumento da relação núcleo/citoplasma. Nestas lesões, ocorre uma desregulação progressiva do ciclo celular pelo HPV, que resulta em aumento da proliferação celular, diminuição ou parada da maturação epitelial e uma menor taxa de replicação viral, com células

escamosas atípicas imaturas expandidas para dois terços da espessura epitelial.

O carcinoma escamoso do colo do útero apresenta alterações características, tais como presença de um número reduzido de células com nucléolos proeminentes, cromatina grosseiramente irregular com aspecto de "tinta Nankin" e diátese tumoral. São compostos por ninhos e projeções de epitélio escamoso maligno, queratinizado ou não, invadindo o estroma cervical subjacente. Este é o segundo tumor maligno mais comum em mulheres no mundo, com aproximadamente 500.000 novos casos diagnosticados responsáveis por 250.000 mortes registradas a cada ano[30,31], e seu desenvolvimento é um processo de múltiplas etapas que envolvem estágios precursores pré-invasivos[14]. Ostör[32] enfatizou o conhecimento de que o carcinoma do colo uterino evolui a partir da lesão precursora incipiente, embora nem todas as lesões evoluam para carcinoma invasivo, visto que algumas lesões epiteliais regridem e em parte dos indivíduos expostos, o HPV permanece inativo na forma latente por um longo prazo[33]. Essa evolução depende também dos fatores de risco relacionados a características tanto do hospedeiro quanto do vírus, como exposição ao HPV, oncogenicidade viral, ineficiência da resposta imunológica, múltiplos parceiros sexuais, idade precoce na primeira relação sexual, imunossupressão, uso de contraceptivos orais e tabagismo[25].

Marcadores de Progressão das Lesões Cervicais

Embora o genótipo viral seja importante marcador para progressão das lesões do colo do útero, outros preditores têm sido estudados para determinar

o resultado clínico, principalmente de mulheres com LIEBG e infectadas por genótipos virais de alto risco oncogênico[34]. Dentre esses marcadores, o estado de incorporação do HPV e o aumento da carga viral relacionado com severidade das lesões cervicais têm sido destaque em diversos estudos[34-37].

O genoma do HPV pode existir em duas formas físicas: uma forma circular fechada episssomal ou linearizadas e integrado ao genoma do hospedeiro[38]. A forma episssomal do vírus é normalmente encontrada na infecção comum ou nas lesões pré-neoplásicas, já na maioria dos carcinomas, observa-se que o HPV encontra-se integrado ou não ao DNA da célula hospedeira[35,39-41]. Este estado de integração do vírus leva a disrupção ou deleção da ORF E2, resultando na falta de expressão da respectiva proteína e consequente *up-regulation* das proteínas E6 e E7 que contribui para progressão das lesões e para a imortalização das células infectadas[14,15,34]. Nesse sentido, a integração dos genótipos de alto risco oncogênico tem sido considerada um marcador para a progressão ao câncer cervical[42].

Em relação à carga viral, Van Duin et al.[43] encontraram que mulheres com citologia normal mas com carga viral superior a $2.4x10^4$ cópias tem risco aumentado para desenvolverem LIEAG e entre as mulheres com citologia anormal, aquelas com carga viral superior a $4.3x10^6$ cópias apresentam risco elevado para LIEAG. Nessa mesma linha, Monnier-Benoit et al.[44] encontraram aumento na incidência de LIEAG, de 14% em mulheres com carga viral inferior a $2.0x10^4$ células para 48% em mulheres com carga superior a esse limite. Entretanto, alguns estudos em relação a esses outros marcadores ainda são controversos[14,45,46] e há tentativa expressiva de se estabelecer um limite para a carga viral a fim de avaliar os riscos de progressão dessas lesões.

Progressão de Lesões Cervicais e Resposta Imune

A resposta imune tem um papel importante na história natural da infecção pelo HPV na cérvice uterina, tanto na persistência viral como no desenvolvimento do câncer do colo do útero[47,48]. As citocinas são proteínas secretadas pelos leucócitos e outras células do organismo em resposta a diferentes estímulos e atuam como mediadoras das células do sistema imune sendo importantes reguladores da transcrição do HPV[49]. Interleucinas (IL) como IL-2, IL-12 e interferon (IFN-γ) encontram-se entre as citocinas produzidas por células Th1, sendo esta associada à resposta mediada por células, particularmente em resistência a patógenos intracelulares, enquanto IL-4, IL-5, IL-6, IL-10 e IL-13 são citocinas de perfil Th2, que estão envolvidas na resposta humoral e produção de imunoglobulina E[50-52].

Muitos estudos sugerem que o padrão Th1 contribui para o estímulo da imunidade celular contra o desenvolvimento da neoplasia associada à infecção pelo HPV e está relacionado com *clearance* dessa infecção, enquanto que o padrão Th2 está associado com a persistência viral da infecção e com o desenvolvimento de neoplasias[53-55]. Tais estudos assumem ainda que o sistema imune do hospedeiro e em particular, a resposta imune local do trato genital inferior, é importante para a sobrevivência do HPV, relacionando-o à neoplasia do colo do útero.

Sharma et al.[52] relataram que, em sobrenadante de cultura de células mononucleares do sangue periférico, o nível de IL-2 esteve significantemente reduzido de acordo com a severidade da lesão cervical e em mulheres com carcinoma invasor, enquanto que o nível de IFN-γ esteve reduzido somente em pacientes com câncer do colo do útero. Ainda nesse estudo, níveis elevados

de IL-4 e IL-10 foram detectados em todas as pacientes com câncer cervical e com lesão intraepitelial escamosa de alto grau, quando comparados com lesão intraepitelial escamosa de baixo grau e com pacientes controles, sugerindo que o padrão Th1 e Th2 de citocinas estiveram significantemente correlacionados com a presença do HPV. Nessa mesma linha, Tsukui et al.[56] demonstraram que o padrão de citocinas Th1, especialmente a produção de IL-2, em resposta ao HPV16 está diminuída em pacientes com lesão intraepitelial escamosa de alto grau e câncer do colo do útero comparada com mulheres com citologia normal. Estes achados são consistentes com a hipótese de que a produção de citocinas protetoras da imunidade mediada por células é deficiente em mulheres com infecção por HPV e que a progressão para lesões precursoras do câncer do colo do útero pode estar associada com a alteração do padrão Th1 para Th2, com a produção de citocinas imuno-regulatórias. Por outro lado, Nguyen et al.[57] descreveram que citocinas de padrão Th1 (IL-2, IFN-γ, TNF-α) e do tipo Th2 (IL-4, IL-5 e IL-10) não foram induzidas significantemente no conteúdo vaginal de pacientes com câncer cervical em relação à ausência dessa condição.

Bais et al.[58] investigando o efeito da infecção pelo HPV sobre a capacidade de produção de citocinas em cultura de células mononucleares do sangue periférico durante a carcinogênese do carcinoma cervical, observaram que as relações Th1/Th2 diminuíram com a progressão das lesões intra-epiteliais cervicais (NIC II para NIC III) e aumentaram de NIC III para carcinoma invasor. Segundo esses autores, significantes mudanças na cinética de produção de citocinas na resposta imune tipo Th2 no soro de mulheres com displasia cervical ocorrem progressivamente do estágio de NIC II para NIC III.

Tijong et al.[59] estudando os níveis de citocinas no lavado cérvico-vaginal de mulheres com neoplasia intraepitelial cervical e com câncer cervical, relataram que os níveis de IL-12 p40, IL-10, TGF-β, TNF-α e IL-1β estavam significantemente elevados em pacientes com câncer cervical em relação às lesões precursoras, demonstrando alterações no ambiente imune cervical local em pacientes com câncer cervical.

Song et al.[47] utilizando a técnica de PCR em tempo real para quantificar IFN-γ, IL-10, IL-6 e TNF–α em cortes histológicos com lesão intraepitelial escamosa de baixo grau, sugeriram que o IFN-γ intralesional pode ser considerado um marcador para *clearance* de HPV de alto risco após 12 meses de *follow-up*. Ainda segundo esses autores, outros fatores como idade, níveis de IL-10, IL-6 e TNF-α, hábito tabagista e uso de anticoncepcional oral não estiveram associados significantemente com HPV de alto risco positivo ou negativo, após 12 meses de *follow-up* em pacientes com lesão intraepitelial escamosa de baixo grau não tratadas.

Já está bem estabelecida na literatura a importância do estudo do papel das citocinas no desenvolvimento do câncer do colo do útero, tendo importantes implicações nas terapias imunes e estratégias de vacinação, por isso o estudo do padrão das citocinas no soro e na secreção cervical, bem como a avaliação da imunomarcação das mesmas nas biópsias cervicais poderá aumentar o conhecimento da imunologia da infecção relacionada ao HPV. Além do mais, considerando a associação do genótipo, carga viral e estado de integração do HPV com a progressão das lesões cervicais e resultados contraditórios de muitos estudos, a análise desses marcadores do desenvolvimento do câncer se justifica.

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3. Resumo

Introdução: A infecção por genótipos de alto risco doPapilomavírus Humano (HPV) está associada ao desenvolvimento das lesões cervicais pré-invasivas e do carcinoma cervical invasor. A carga viral e o estado físico desses genótipos de HPV são considerados importantes biomarcadores para a progressão dessas lesões. A resposta imune do hospedeiro tem um papel importante na história natural da infecção pelo HPV na cérvice uterina como, por exemplo, a persistência viral e o desenvolvimento do câncer cervical. As citocinas são os principais mediadores desta resposta imune e são capazes de regular a transcrição do HPV dependendo do padrão de resposta ao qual estão associadas. O padrão Th1 contribui para o desenvolvimento da imunidade celular contra a infecção pelo HPV e está relacionado com *clearance* dessa infecção.Por outro lado, o padrão Th2 está associado com a persistência viral da infecção e com a progressão das lesões cervicais. A maioria dessas infecções provavelmente envolve um balanço entre osperfis Th1 e Th2 da resposta imune. **Objetivo**: Avaliar a concentração de IL-1β, IL-4, IL-6, IL-10, TNF-α e INF-γ na secreção e no soro e a intensidade da imunomarcação destas citocinas nas biópsias cervicais. Além disso, determinar a carga viral e o estado físico do HPV16 em mulheres portadoras de lesão intraepitelial escamosa de baixo grau (LIEBG), lesão intraepitelial escamosa de alto grau (LIEAG), carcinoma cervical invasor (CCI) e no grupo controle.**Pacientes e Métodos**: Foram incluídas no estudo 109 mulheres com diagnóstico histopatológico de LIEBG (n=16), LIEAG (n=40), CCI (n=13) e 40 mulheres sem alterações histopatológicas na biópsia do colo do útero (grupo controle), atendidas no Ambulatório de Ginecologia Preventiva do Hospital Amaral

Carvalho, Jaú, SP. As concentrações dasdiferentes citocinasno soro e na secreção cervical foram determinadas por ensaio imunoenzimático (ELISA). Para avaliar a imunomarcação dascitocinas nas biópsias cervicais foi utilizada a técnica de imunohistoquímica. A detecção e a genotipagemdo HPV nos fragmentos das biópsias foram realizadas pela técnica de reação em cadeia da polimerase (PCR) e por Linear Array® HPV (Roche Molecular Diagnostics), respectivamente. Para determinar a carga viral e do estado físico do HPV16, foi realizada a técnica de PCR em tempo real utilizando *primers* específicos e, para esta análise, foram incluídas 57 mulheres positivas para DNA de HPV16 com LIEBG (n=10), LIEAG (n=35) e um grupo controle (n=12). **Resultados:** DNA-HPV foi detectado em 90% das amostras do grupo controle, 93,7% no grupo LIEBG, 100% no grupo LIEAG e 84,6% no grupo CCI. O genótipo HPV16 foi prevalente em todos os grupos (63,3%), seguido por HPV18 (11,2%), HPV31 (7,2%), HPV33 (6,1%), HPV52 (4,1%), HPV58 (4,1%) e HPV tipos 26, 40, 42, 45, 53 e 66 (1,0% cada). A infecção mista (diferentes genótipos de HPV) foi observada em 15,3% das lesões. Considerando a carga viral relativa de HPV16, não foi observada diferença significativa entre o grupo controle e os grupos LIEBG e LIEAG [(61,2 (0-584,7), 191,8 (9-1154,3) e 114,7 (4,3-1.118,3)], respectivamente. O estado físico do HPV na forma episssomal foi maior no grupo controle (66%) em comparação ao grupo LIEAG (22%) (p=0,009). No entanto, não houve diferença estatisticamente significativa quando comparado com o grupo LIEBG (30%) (p≥ 0,05). Em relação à integração do DNA de HPV16, a proporção de HPV16 misto e integrado aumentouconforme a gravidade da lesão (p=0,018). Os níveis das citocinas IL-4, IL-6 e IL-10 na secreção cervical estavam significantemente maiores nas

pacientes com CCI, quando comparado com o grupo controle e os grupos LIEBG e LIEAG ($p<0,05$). Não foram observadas diferenças em relação aos níveis de IL-1β, TNF-α e IFN-γ entre os grupos estudados ($p>0,05$). Considerando os níveis dessas citocinas no soro, a IL-6 estava significantemente aumentada nas pacientes com LIEAG em comparação aosoutros grupos ($p<0,05$).Não foram observadas diferenças nos níveis de IL-1β, IL-4, IL-10, TNF-α e IFN-γ entre os grupos estudados ($p>0,05$). Em relação à intensidade de imunomarcação, a expressão de IFN-γ foi maior no grupo controle quando comparado com os grupos LIEBG, LIEAG e CCI ($p<0,001$). Já a expressão de IL-4 foi maior no grupo LIEAG quando comparado ao grupo controle ($p=0,029$). A intensidade de imunomarcação de IL-1β, IL-6, IL-10 e TNF-α foi semelhante em todos os grupos, não havendo diferença estatisticamente significativa ($p\geq0,05$). **Conclusão**: Nossos dados sugerem que a alta frequência de integração dos genótipos de alto risco de HPV mostra seu potencial maligno na progressão das lesões e que a carga viral não parece ser um importante biomarcador para essa progressão. Além disso, nosso estudo sugere que o aumento de citocinas do padrão Th2 em mulheres com LIEAG e CCI está relacionado com a progressão para o carcinoma cervical invasor.

4. Abstract

Introduction: Infection with high-risk genotypes of the human papillomavirus (HPV) is associated with the development of preinvasive cervical lesions and invasive cervical carcinoma. The viral load and physical state of HPV genotypes are considered important biomarkers for lesion progression. The immune response has an important role in the natural history of HPV infection in the uterine cervix, as, for example, the viral persistence and the development of cervical cancer. Cytokines are the main mediators of this immune response and they regulate the HPV transcription depending on the response pattern to which they are related. The Th1 pattern contributes to the development of cellular immunity against HPV infection and its clearance. On the other hand, Th2 pattern is associated with the persistence of viral infection and the progression of cervical lesions. Most of these infections probably involves a balance between Th1 and Th2 immune response profiles.**Objective:** To evaluate the concentration of IL-1 β, IL-4, IL-6, IL-10,TNF-α and INF-γ in secretionand serum and also to assess the immunostaining intensity of these cytokines in cervical biopsies.Moreover, the present study aimed to determine the viral load and physical state of HPV16 in women with low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), invasive cervical carcinoma (ICC) and also in the control group.**Patients and Methods:** This study included 109 women with histopathologic diagnosis of LSIL (n=16), HSIL (n=40), ICC (n=13) and 40 women with no pathological changes on cervix biopsy (control group) attended in Preventive Gynaecological Ambulatory at the Amaral Carvalho Hospital, Jaú, São Paulo, Brazil. Cytokine concentrationwas determined in serum and cervical secretion by enzyme-linked immunosorbent

assay (ELISA). Immunohistochemistry was used to evaluate the immunostaining intensity of cytokines in biopsy fragments. HPV detection and genotyping in biopsy fragmentswere performed by polymerase chain reaction (PCR) and Linear Array® HPV (Roche Molecular Diagnostics), respectively. To determine the viral load and the physical state of HPV16, real-time PCR using specific primers was performed and, for this analysis, we included 57 HPV16 DNA positive women with LSIL (n=10) and HSIL (n=35) and, also, a control group (n=12). **Results:** HPV DNA was detected in 90.0% in control group, 93.7% LSIL, 100.0% HSIL and 84.6% ICC. HPV16 genotype was the most prevalent in all groups (63.3%) followed by HPV 18 (11.2%), HPV31 (7.2%), HPV33 (6.1%), HPV52 (4.1%), HPV58 (4.1%) and HPV types 26, 40, 42, 45, 53 and 66 (1.0% each). Mixed infections (different HPV genotypes) were observed in 15.3% of the lesions. Considering the relative viral load of HPV16, no significant difference was observed among control group and LSIL and HSIL groups [(61.2 (0-584.7), 191.8 (9-1154.3) and 114.7 (4.3- 1118.3)], respectively. HPV physical state in episomal forms was higher in control group (66.0%) compared with HSIL group (20.0%) ($p=0.009$).However, there was no statistically significant difference when compared with LSIL group (30.0%) ($p \geq 0.05$). Considering the HPV16 DNA integration, the proportion of mixed and integrated HPV16 increased towards the severity of the lesion ($p=0.018$). IL-4, IL-6 and IL-10 levels in cervical secretion were significantly increased in patients with ICC when compared with the control group and the LSIL and HSIL groups ($p<0.05$). No differences were observed regarding IL-1β, TNF-α and IFN-γ levels among the groups ($p>0.05$). Considering the cytokine levels in the serum, IL-6 level was significantly increased in patients with HSIL when

compared withthe other groups ($p<0.05$). No differences were observed regarding IL-1β, IL-4, IL-10, TNF-α and IFN-γlevels among the groups ($p>0.05$). In relation to immunostaining intensity, IFN-γ expression was higher in the control group when compared with LSIL, HSIL and ICC groups ($p<0.001$). IL-4 expression was higher in HSIL in comparison to the control group ($p=0.029$). The immunostaining intensity of IL-1β, IL-6, IL-10 and TNF-α was similar in all groups, with no statistically significant difference ($p\geq0.05$).**Conclusion:** Our data suggest that the high frequency of integrated HR-HPV show their malignant potential to the lesion progression and also, that the viral loaddo not seem to be an important biomarker for this progression.Our study also suggests that the increase of Th2 cytokines in women with HSIL and ICC is related to the progression of cervical lesion.

Viral load and physical state of Human Papillomavirus type 16 in women with preinvasive cervical lesions.

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Abstract

Background: High-risk HPV genotypes persistence, especially 16, are associated with tumour progression from low-grade squamous intraepithelial lesion (LSIL) to an invasive cervical carcinoma (ICC) via a high-grade squamous intraepithelial lesion (HSIL). Viral load and physical state of those HPVs types have been considered useful biomarkers for this disease progression. The aim of this study was to determine the viral load and physical state of HPV16 in preinvasive cervical lesions.

Methods: A molecular study was conducted with 56 women with histopathologic diagnosis of LSIL (n=16) and HSIL (n=40). The control group consisted in women with no pathological changes on cervix biopsy (n=40). All subjects were attended at Preventive Gynaecological Ambulatory at the Amaral Carvalho Hospital, Jaú, São Paulo, Brazil. The detection and HPV genotyping in the fragments of the biopsies were performed by PCR and Linear Array® HPV (Roche Molecular Diagnostics), respectively. To determine the viral load and the physical state of HPV16, real-time PCR using specific primers was performed and, for this analysis, we included 57 HPV16 DNA positive women with LSIL (n=10) and HSIL (n=35) and, also, a control group (n=12).

Results: HPV DNA was detected in 90.0% in control group, 93.7% in LSIL and 100.0% in HSIL women. The HPV type 16 was the most prevalent in all groups (59.3%). Considering the relative viral load of HPV16, no significant difference was observed among control group, LSIL and HSIL [(61.2 (0-584.7), 191.8 (9-1154.3) and 114.7 (4.3- 1118.3)], respectively. HPV physical state in episomal forms was higher in control group (66.0%) compared with HSIL (20.0%) ($p=0.009$), but there was no statistically significant difference in comparison to LSIL (30.0%) ($p \geq 0.05$). Considering the HPV16 DNA integration, the proportion of mixed and integrated HPV16 increased towards the severity of lesion ($p=0.018$). **Conclusion:** Our data suggest that the high frequency of integrated HR-HPV shows their malignant potential to lesion progression and that the viral load do not seem to be important biomarker for preinvasive cervical lesion progression.

Keywords: HPV16, viral load, physical state, cervical lesion progression.

Background

Cervical carcinoma is the second most common malignancy tumor in women worldwide, with approximately 500,000 new cases diagnosed with an annual mortality rate of 250,000[1,2].

The development of this disease is a multistep process that also involves preinvasive lesions[3]. High-risk HPV genotypes (HR-HPV) persistence is associated with cervical precancerous lesions and the development of invasive cervical carcinoma. Consequently, HR-HPV infection has been considered as a marker for the progression of low-grade squamous intraepithelial lesion (LSIL) to an invasive cervical carcinoma (ICC) via a high-grade squamous intraepithelial lesion (HSIL)[3]. However, studies from the past decades showed that other predictors may also contribute to this transformation, such as the viral load and the physical state of HPV[3-8]. Even though some studies have shown an association of viral load increase with severity of cervical lesions and integration state of the HPV genome in the host cell DNA[5,7,9], other studies are controversial[3,10]. For this reason, there is still a significant attempt to establish a limit for the viral load in order to evaluate the risks of lesion progression.

The HPV genome exists in two physical forms: a circular form, episomal, or linear and integrated to the host genome[8]. The episomal form is usually found in common infection or precancerous lesions. Nonetheless, it is observed that in most cancers HPV is integrated in the host cell DNA [9-12]. The virus integration status leads to deletion or disruption of the E2 ORF region, resulting in loss of function and consequent overexpression of viral proteins E6 and E7. These proteins promote the disruption of the cell cycle through interaction with important regulators of the cellular proliferation, such as p53 and Rb, contributing to the progression of the lesions and the immortalization of infected cells[3-5]. In fact, the integration of oncogenic HR-HPV has been considered an important marker for the cervical cancer progression[4,9,13]. However, some studies showed the coexistence of episomal and integrated forms not only in HSIL and ICC, but also in LSIL and normal cervical epithelium[10,14].

Considering those contradictory findings, this study aimed to determine the viral load and physical state of HPV 16 in preinvasive cervical lesions.

Methods

Subjects

We conducted a prospective and cross-sectional study, which enrolled 56 women with histopathologic diagnosis of LSIL (n = 16) and HSIL (n = 40). The control group consisted in women with no pathological changes on cervix biopsy (n=40). All subjects were attended at Preventive Gynaecological Ambulatory at the Amaral Carvalho Hospital, Jaú, São Paulo, Brazil, from March 2011 to July 2014.

All the patients in the study were previously explained about the purpose of the research and written informed consent was obtained. This study was approved by the Ethics Research Committee of Botucatu Medical School, São Paulo State University (Protocol 3162-2009) and by the Ethics Commitee of the Amaral Carvalho Hospital, Jaú, São Paulo, Brazil (Protocol 181/09).

Sampling procedures

Following the standard routine, during colposcopic exam of the cervix, fragments of cervical biopsies were obtained for HPV detection and genotyping by polymerase chain reaction (PCR). Samples were collected in 500μl of Tris-EDTA-Tween solution and stored at -20°C until analysis.

Histopathological analysis

The lesion fragments were fixed in 10% formalin for 24 hours, dehydrated with alcohol, diaphanized in xylene and finally paraffin-embedded. The blocks were sectioned in 6μm slides and stained with Hematoxylin & Eosin.

The biopsies were classified according to the Bethesda system[15] in normal epithelium, LSIL and HSIL (Figure 1). Normal epithelium was characterized by show

keratinocytes with light halo ill-defined, featuring the presence of glycogen, in the superficial part (Figure 1A); LSIL was characterized by the presence of mild koilocytosis in the upper third and disorganization of the lower third of the epithelium (Figure 1B); and HSIL that was characterized by the presence of koilocytosis, nuclear and cellular abnormalities, disorganization of the lower half of the epithelium and superficial parakeratosis (Figure 1C).

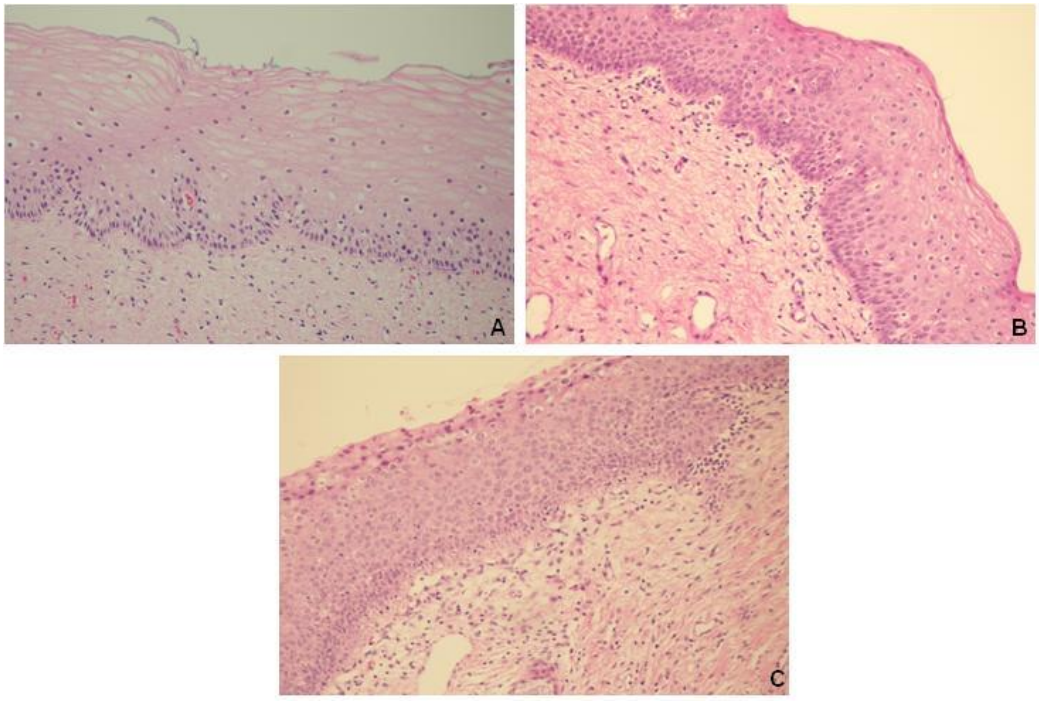

Figure 1. Photomicrography of cervix showing normal epithelium (A), Low-grade squamous intraepithelial lesion (B) and High-grade squamous intraepithelial lesion (C).

HPV DNA detection and genotyping

DNA extraction was performed using the Illustra Kit (GE Healthcare, Barrington, IL, USA) according to the manufacturer's instructions and DNA integrity was confirmed by the amplification of β-globin constitutive gene, using the primers PCO4 and GH20[16]. The initial HPV detection was performed by nested PCR using, first, a pair of L1 consensus degenerate primers MY09/11[17] and, then, amplification of 2μL of the primary PCR product using the primers GP5+/6+[18]. PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI, USA), according to the manufacturer's

instructions. Each PCR reaction included negative (ultrapure water) and positive (HPV DNA extracted from HeLa cells) controls. Amplified product was determined by comparison with a standard size marker under electrophoresis on a Gel Red™ agarose gel.

HPV genotyping was performed by Linear Array Genotyping Test (Roche Molecular Diagnostics, Indianapolis, IN, USA) which is able to identify 37 HPV genotypes. Reactions were amplified in Eppendorf Mastercycle Personal thermal cycler and were performed according to the manufacturer's instructions. A positive and negative control, included in the kit, was used in each assay. HPV16 positive samples were subjected to analysis of viral load and physical state, we included 57 HPV16 DNA positive women with LSIL (n=10), HSIL (n=35) and, also, a control group (n=12).

HPV16 viral load

Relative quantification of viral load in HPV16 DNA positive samples was performed by real-time PCR, which was used a 25μL final volume reaction with Maxima SYBR Green/ROX (Fermentas, St. Leon-Rot, Germany) and 2μL of sample, for amplification of a 217bp sequence, using the primers HPV16 forward (5´-CGCACAAAACGTGCATCGGCTACC-3´) and HPV16 reverse (5´-TGGGAGGCCTTGTTCCCAATGGA-3´)[19] in a concentration of 10μM. Assays were run on the ABI Prism 7300 (Applied Biosystems, Carlsbad, CA, USA) in 40 cycles of amplification and all reactions were performed in duplicate and negative controls were included in each run. Relative viral load was calculated through the normalization against the concentrations of β-globin DNA and interpolation of cycle threshold (Ct) from each sample to a standard curve constituted by serial dilution of the plasmid DNA (pBR322-HPV16) kindly donated by Dr. Joakim Dillner (International HPV Reference Center - Karolinska Institute, Stockholm, Sweden).

Physical state of HPV16 genome

To determine the physical state of the viral genome in cervical lesions genotyped with HR-HPV16 was performed by real-time PCR using amplifications of specific primers and probes targeting the viral genes E2 and E6 open reading frames regions (Table 1).

Table 1. Primers and probes used for real-time PCR

Primer	Sequence (5´- 3´)	Tm (°C)	Amplicon size (bp)
Probe 16E2PRO	(5HEX)-CACCCCGCCGCGACCCATA-(BHQ1)	70	
Primer 1, 16E2F	AACGAAGTATCCTCTCCTGAAATTATTAG	59	82
Primer 2, 16E2R	CCAAGGCGACGGCTTTG	60	
Probe 16E6PRO	(6-FAM)-CAGGAGCGACCCAGAAAGTTACCACAGTT-(BHQ1)	69	
Primer 1, 16E6F	GAGAACTGCAATGTTTCAGGACC	59	81
Primer 2, 16E6R	TGTATAGTTGTTTGCAGCTCTGTGC	60	

F: forward; R: reverse; BHQ: Black Hole Quencher; Tm: melting temperature

Initially, 2μL of each sample (100ng/μL) was amplified with 1μL of each primer and probe in a 10μM concentration, in a 25μL final volume reaction using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA). ABI Prism 7300 instrument thermal cycling (Applied Biosystems, Carlsbad, CA, USA) was initiated with a denaturation step of 10 min at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Then, the E2/E6 ratio was calculated for each sample to determine viral integration of HPV16 DNA, wherein E2/E6 = 0 representing the full integration of the HPV 16 genome; E2/E6 ≥ 1 represented predominantly episomal viral genome and E2/E6 > 0 and < 1 indicated a mixed form (integrated + episomal). For this, a standard curve was used as control, consisting of serial dilutions of pBR322-HPV16 plasmid. All samples and standard curves were tested in duplicate and a negative control was included in every run, the data were analyzed in 7300 System SDS Software version 1.2.3. (Applied Biosystems, Carlsbad, CA, USA).

Statistical analysis

The sample size of 40 women for each group was calculated based on the estimated variability on the quantity viral load and frequency of HPV integration assuming α=0.05 and β=0.20. The comparison of relative quantification of HPV16 viral load towards the magnitude of lesion was assessed by the Jonckheere-Terpstra trend test and the association between the HPV16 physical state and the severity of the lesions was estimated by the Linear-to-linear association test. Data was analyzed by IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA) and the significance was set as $p<0.05$.

Results

Detection and genotyping of HPV DNA

HPV DNA was detected in 90.0% of samples from the control group, 93.7% in LSIL and 100.0% in HSIL. HPV16 genotype was the most prevalent in all groups (59.3%), followed by HPV 18 (10.2%), HPV31 (6.2%), HPV33 (5.1%), HPV52 (4.1%), HPV58 (3.1%) and HPV types 26, 40, 42, 45, 53 and 66 (1.0% each). The mixed infections with different HPV genotypes were observed in 15.3% of the lesions (Figure 2).

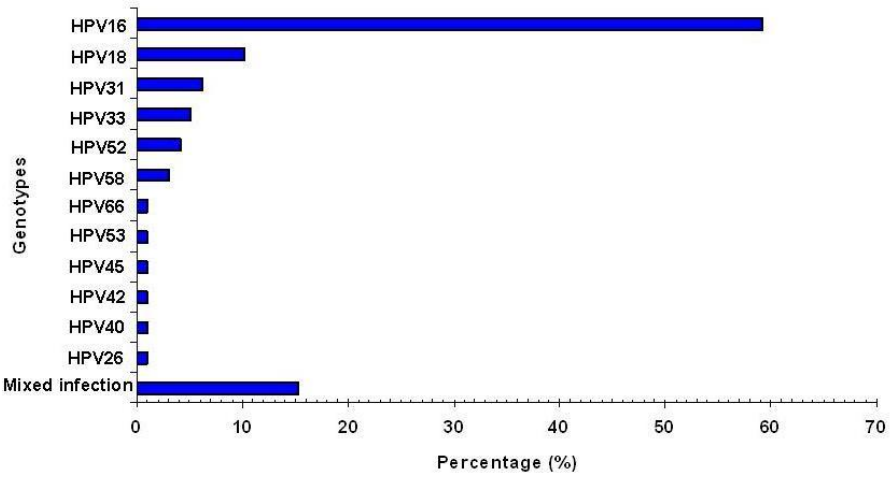

Figure 2. Prevalence of HPV genotypes evaluated in cervical biopsies in patients with LSIL, HSIL and control group.

Relative quantification of HPV16 viral load

Relative viral load was determined in 57 HPV16 DNA positive samples: control group (n=12), LSIL (n = 10) and HSIL (n = 35). Considering the viral relative quantification, viral load doesn´t differ among grades of lesion in this samples (p=0.18). (Figure 3).

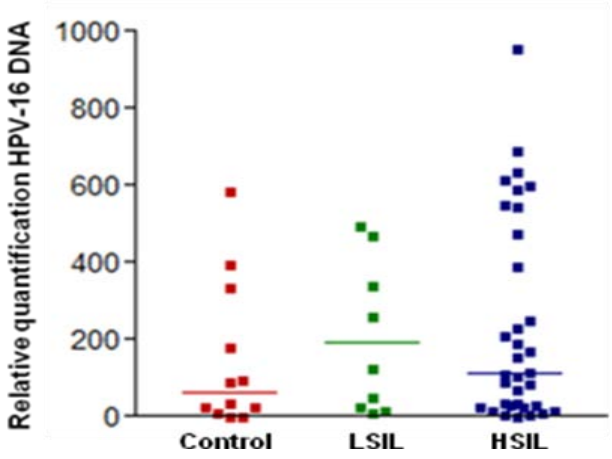

Figure 3. Dot-plot representing the relative quantification of viral load detected in samples positive for HPV16 DNA included in the study regarding the studied groups. Jonckheere-Terpstra trend test (p> 0.05).

Determination of HPV16 physical state

The analysis of the E2/E6 ratio for HPV16 physical state evaluation in all positive samples for this viral type, demonstrated that the episomal form of HPV was higher in control group compared with HSIL (p=0.009), but there was no statistically significant difference when compared with LSIL (p≥0.05). Considering the HPV16 DNA integration, the proportion of mixed and integrated HPV16 increased towards the severity of lesion (p=0.018).

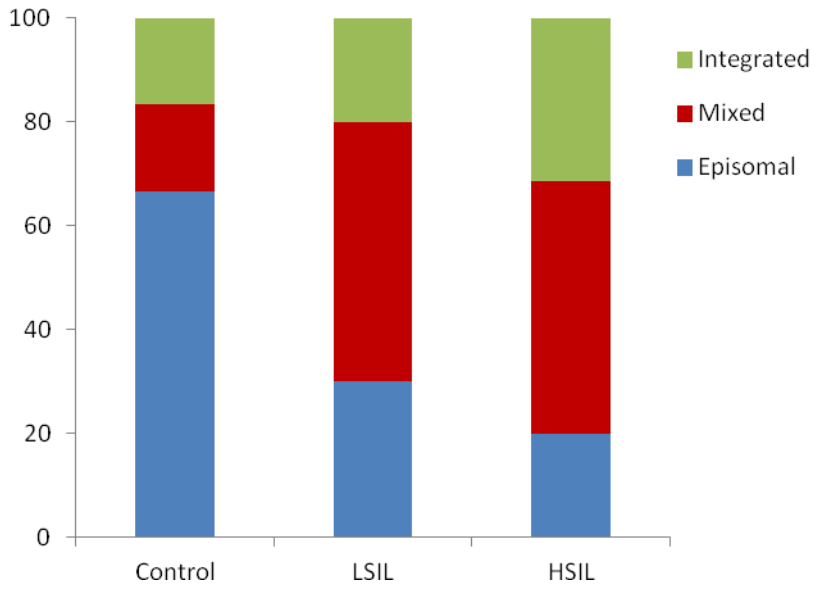

Figure 3. Proportion of the physical status of HPV16 DNA in relation to the groups. Linear-to-linear association test ; p=0.018

Discussion

HPV cervical infection is the main precursor of a series events that lead to cervical cancer and it has been thoroughly documented in epidemiological and experimental studies during the last years[1,2,13]. In the present study, HPV DNA was detected in more than 90.0% of the samples in all groups, which corroborates with previous studies that found a high prevalence of HPV infection in women without cervix alterations[20] and HPV prevalence of 99.0 to 100.0% in women who had lesions[21].

High risk genotypes persistence is associated with intraepithelial lesion progression[3,22,23]. Some studies have demonstrated the association of increased viral load with a persistent infection[24,25] and with an increased risk for high-grade cervical lesions and cancer development[26-28]. However, some other studies are still contradictory[3], finding a weak association[29] or lower HPV16 viral load in high-grade compared with low-grade lesions[30]. In this cross-sectional study, there was no significant difference in the relative quantification of HPV16 DNA in the samples considering the evaluated groups. This finding is supported by studies that did not observe association between viral load and severity of cervical lesion[31,32]. For this

reason, there is a significant attempt to establish a viral load limit for the purpose of evaluate the risks of the progression of these lesions. We are aware of this study limitation, because it did not reach the sample size calculated. However, the posthoc analysis produced a satisfactory power test based on the viral load of HPV 16. Thus, this sample size limitation does not hamper our findings.

The HPV DNA incorporation state in the cellular genome is also related to the lesion progression. This integration is associated with HSIL and cancer, as reported in several studies that demonstrated increased in samples with integrated forms proportionally the cancer progression[4,6,7,9,10,31-34]. Our findings are in agreement with these studies, since we observe a higher frequency of episomal forms in the control group compared with HSIL and proportion of mixed and integrated HPV16 increased towards the severity of lesion.

However, the integration of oncogenic HR-HPV is not always necessary for the cancer development, since there are HSIL and carcinomas with no HPV DNA integration[14,35,36]. Moreover, Shukla et al.[37] demonstrated different HPV16 variants and small variations in its sequence expressing variable frequencies of different physical status forms. Early integration of HPV16 DNA in LSIL and normal cervical samples, as we found in our study, was described by several researchers[9,10,38,39]. Accordingly, Van Tine et al.[40] demonstrated that DNA methylation is partially responsible for transcription silencing of viral DNA integrated copies and although has been observed that an accumulation of integrated genome in few loci, a integration hot spot could not be identified, since viral integration seems to be related with these genomic fragile areas than the regions of the tumor relevant genes[41], showing that HR-HPV viral integration does not seem be a prerequisite for the cervical cancer development.

Conclusion

This study demonstrated an increase in the HPV16 episomal forms in control group and that mixed and integrated HPV16 frequency increases with the severity of the lesion, but no difference was found in viral load quantification in the lesion progression. So, our data suggest that the high frequency of integrated HR-HPV shows their malignant potential to lesion progression and that the viral load do not seem to be important biomarker for preinvasive cervical lesion progression.

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Abbreviations

HPV: Human papillomavirus; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intra-epithelial lesion; ICC: invasive cervical carcinoma; HR HPV: High risk types of HPV; PCR: Polymerase chain reaction; ORF: open reading frames

Competing interests The authors declare that they have no competing interests.

Authors' contributions

LDM analyzed and interpreted the data and drafted the manuscript; LMQM collected all the samples; MGS participated in the study design, interpreted the data and supervised the study. All authors read and approved the final manuscript.

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Cytokine profile in women with preinvasive cervical lesions and invasive cervical carcinoma.

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Abstract

Introduction: Persistence of high-risk HPV genotype is a marker for tumour progression from low-grade squamous intraepithelial lesion (LSIL) to an invasive cervical carcinoma (ICC) via a high-grade squamous intra-epithelial lesion (HSIL). Most HPV infections likely involve a balance of Th1 and Th2 immune response types. Objective: The aim of this study was to evaluate the concentration of IL-1β, IL-4, IL-6, IL-10, TNF-α and IFN-γ in serum, cervical secretion and biopsies of women with LSIL, HSIL, ICC and control group. Patients and Methods: The study included 109 women with LSIL (n=16), HSIL (n=40), ICC (n=13) and 40 women with no pathological changes on cervix biopsy (control group). The cytokine concentrations were determined in cervical secretion and serum by enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry was used to evaluate immunostaining cytokine intensity in biopsy fragments. HPV detection and genotyping in biopsy fragments were performed by polymerase chain reaction (PCR) and Linear Array® HPV (Roche Molecular Diagnostics), respectively. Results: HPV DNA was detected in 90.0% in control group, 93.7% LSIL, 100.0% HSIL and 84.6% ICC. The HPV type 16 was the prevalent in all groups. IL-4, IL-6 and IL-10 levels in cervical secretion were significantly increased in patients with ICC compared with control group, LSIL and HSIL groups ($p<0.05$) and no differences were observed regarding IL-1β, TNF-α and IFN-γ levels among the studied groups ($p>0.05$). In the serum, IL-6 levels were significantly increased in patients with HSIL compared with control group, LSIL and ICC groups ($p<0.05$) and no differences were observed in IL-1β, IL-4, IL-10, TNF-α and IFN-γ levels among the studied groups ($p>0.05$). Considering the immunostaining intensity, IFN-γ was higher in control group when compared with LSIL, HSIL and ICC groups ($p<0.001$) and IL-4 was higher in HSIL when compared with control group ($p=0.029$). IL-1β, IL-6, IL-10 and TNF-α immunostaining intensity means were similar in all groups, with no statistically significant difference ($p\geq0.05$). Conclusion: These results suggest that the increase of Th2 cytokines in women with HSIL and ICC is related to the cervical lesion progression.

Keywords: HPV, cytokine concentrations, immunostaining, cervical lesion progression, invasive cervical carcinoma.

Introduction

Human papillomavirus (HPV) infection is an important risk factor for the development of cervical cancer. The persistence of high-risk HPV genotypes (HR-HPV) is associated with the progression of low-grade squamous intraepithelial lesion (LSIL) to an invasive cervical carcinoma (ICC) via a high-grade squamous intraepithelial lesion (HSIL) [1-4]. Although the viral genotype is an important marker for lesion progression, other predictors have been studied to determine clinical outcome, especially in women with LSIL infected by HR-HPV [5-6].

The host systemic and local immune responses are important factors in defining the natural history and the course of HPV infection. Both innate and adaptive immunity are required to recognize and combat viral infections [7-9]. T lymphocytes are the main players of the adaptive immunity, consisting in two major subpopulations: $CD4^+$ and $CD8^+$ T cells [9]. $CD4^+$ T cells can be further subdivided into two main subsets (Th1 and Th2) according to their cytokine profile. Cytokines are proteins secreted by leukocytes and other cells in response to different stimuli. Th1 cytokines (IL-1β, IL-2, IL-12, TNF-α and IFN-γ) induce cell-mediated immune response against intracellular pathogens, inhibit humoral immunity and are related to clearance of HPV infection. On the other hand, Th2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13) induce humoral immune response, inhibit cell-mediated immunity and are associated with viral persistence and lesion progression [10-12]. Several studies showed that the viral persistence is associated with the lack of Th1 pattern cytokine production. Moreover, the progression of preinvasive cervical lesions is associated with a shift from Th1-type to Th2-type immune response [13-16].

Until now, there are a few reports in the literature describing the immune markers on the site of infection and whether systemic measures correlate with local response. In fact, it is difficult to determine the cytokines activity and their involvement in tumor progression [16-18]. Therefore, the aim of this study was to evaluate the concentration of IL-1β , IL-4 , IL-6 , IL-10,

TNF-α and IFN-γ in serum, cervical secretion and biopsies of women with LSIL, HSIL, ICC and control group.

Material and Methods

Patients

From 2011 to 2014, 69 women with histopathologic diagnosis of LSIL (n = 16), HSIL (n = 40) and ICC (n = 13) and 40 women with no pathological changes on cervix (control group) attending in Preventive Gynaecological Ambulatory at the Amaral Carvalho Hospital, Jaú, São Paulo, Brazil were invited to participate of this prospective and cross-sectional study. All subjects were previously informed about the study aims and written informed consent was obtained. This study was approved by the Ethics Research Committee of Botucatu Medical School, São Paulo State University (Protocol 3162-2009) and by the Ethics Commitee of the Amaral Carvalho Hospital, Jaú, São Paulo, Brazil (Protocol 181/09).

Sampling procedures

Following the local protocol, during the specular exam, the cervical secretions were collected with a cytobrush, conditioned in a falcon tube with 2mL of Phosphate Buffered Saline (PBS), centrifuged at 855g and 4ºC for 10 minutes, the supernatant was stored in aliquots at -80ºC and used to determine the cervical cytokine levels by enzyme-linked immunosorbent assay (ELISA). The fragments of cervical biopsies were also collected in 500µl of Tris-EDTA-Tween solution for HPV detection and genotyping by polymerase chain reaction (PCR) and an additional sample were fixed in formalin and paraffin-embedded tissue blocks were sectioned and stained with Hematoxylin & Eosin (HE) to histopathological analysis and were sectioned and mounted in slides to evaluate immunostaining cytokine using immunohistochemistry.

The peripheral venous blood of women included in the study was collected in 10 mL sterile vacutainers (Becton-Dickinson, Meylan, NJ, USA), centrifuged at 1600g for 10 minutes at room temperature and the serum was stored in aliquots at -80 ° C for assessment of cytokine patterns by ELISA.

Histopathological analysis

The biopsies were classified according to the Bethesda system [19] and revised for an experienced pathologist in normal epithelium characterized by show keratinocytes with light halo ill-defined in the superficial part, featuring the presence of glycogen; LSIL characterized by the presence of mild koilocytosis in the upper third and disorganization of the lower third of the epithelium; HSIL characterized by the presence of koilocytosis, nuclear and cellular abnormalities, disorganization of the lower half of the epithelium and superficial parakeratosis and ICC characterized by keratinocytes blocks with atypical cells in the corium in the middle of the lymphocytic infiltrate.

HPV DNA detection and genotyping

DNA extraction was performed using the Illustra Kit (GE Healthcare, Barrington, IL, USA) according to the manufacturer's instructions and DNA integrity was confirmed by the amplification of β-globin constitutive gene, using the primers PCO4 and GH20 [20]. The initial HPV detection was performed by nested PCR using, first, a pair of L1 consensus degenerate primers MY09/11 [21] and, then, amplification of 2μL of the primary PCR product using the primers GP5+/6+ [22]. PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI, USA), according to the manufacturer's instructions. Each PCR reaction included negative (ultrapure water) and positive (HPV DNA extracted from HeLa cells) controls. Amplified product was determined by comparison with a standard size marker under electrophoresis on a Gel Red^{TM} agarose gel.

HPV genotyping was performed by Linear Array Genotyping Test (Roche Molecular Diagnostics, Indianapolis, IN, USA) which is able to identify 37 HPV genotypes. Reactions were amplified in Eppendorf Mastercycle Personal thermal cycler and were performed according to the manufacturer's instructions. A positive and negative control, included in the kit, was used in each assay.

Measurement of cytokine concentration

IL-1β , IL-4 , IL-6 , IL-10, TNF-α and IFN-γ concentrations were measured in cervical secretion and serum by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (DuoSet Kits, R&D Systems, Minneapolis, MN, USA). Briefly, 96-well plate (MaxSorp, Nunc, Life Technologies Incorporation, Maryland, USA) were previously coated with specific capture antibody and then blocked. Samples and standards were incubated for 3 hours at room temperature (RT) and after wash, biotinylated antibody for each cytokine was added and incubated for 2 hours at RT. Substrate solution was added to each well for 30 minutes and then the Stop Solution for 10 minutes. Optical density was measured in a spectrophotometer (Epoch-BioTek) using a 492nm filter. All samples were tested in duplicate and the quantification was based on their mean value. The final concentration was estimated from a standard curve generated from serial dilutions of the recombinant purified cytokine provided in each kit. The intra-assay variability mean rates were 7.4% for IL-1β, 8.9% for IL-4, 9.8% for IL-6, 10.2% for IL-10, 7.9% for TNF-α and 8.1% for IFN-γ. The detection limit of IL-1β, IL-4, IL-6, IL-10, TNF-α and IFN-γ assays were 0.2 pg/mL, 4.2 pg/mL, 0.3 pg/mL, 4.0 pg/mL, 14.6 pg/mL e 7.9 pg/mL, respectively.

Immunohistochemistry

For evaluation of immunostaining of cytokines IL-1β, IL - 4 , IL - 6 , IL - 10, TNF-α and IFN-γ in the biopsies fragments, histological sections from paraffin-embedded blocks were mounted on silanized slides, dried at 37ºC for 24 hours and subjected to deparaffinization and to antigen retrieval with Trilogy™ solution (Cell Marque, Hot Springs, AR, USA). Staining was performed using Mach 4 universal HRP Polymer Kit with DAB (Biocare Medical, Concord, CA, USA) according to the manufacturer's instructions. Sections were counterstained with Harris hematoxylin and mounted. Negative controls were performed for each specimen, in which sections were incubated without the primary monoclonal antibodies. The positive staining was detected as brown staining cytoplasmic pattern. The analysis of the

immunostaining intensity of interest cytokines was conducted from images of histological sections that were captured by a camera attached to the microscope and computer for image scanning, in which three areas with higher immunostaining (hot spots) for each histological section were selected and determined by their areas mean value (pixels^2) using the software Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The sample size of 40 women for each group was calculated based on the estimated variability on the cytokines concentrations in cervical secretion, serum and biopsies fragments assuming α=0.05 and β=0.20. The Kolmogorov-Smirnov test was used to check the normality of the data. The cytokines concentrations in cervical secretion, serum and biopsies fragments were compared between the groups using the nonparametric Kruskall-Wallis test. The statistical analysis was carried out using SigmaStat 3.1 software (Jandel Corporation, San Rafael, CA, USA). The level of significance was set at 5%.

Results

Detection and genotyping of HPV DNA

HPV DNA was detected in 90.0% of samples from the control group, 93.7% in LSIL, 100.0% in HSIL and 84.6% ICC. HPV16 genotype was the most prevalent in all groups (63.3%) followed by HPV 18 (11.2%), HPV31 (7.2%), HPV33 (6.1%), HPV52 (4.1%), HPV58 (4.1%) and HPV types 26, 40, 42, 45, 53 and 66 (1.0% each). The mixed infections with different HPV genotypes were observed in 15.3% of the lesions (Figure 1).

Determination of cytokine concentration in cervical secretion

The analysis of IL-4, IL-6 e IL-10 demonstrated that a significantly higher concentration of these cytokines in patients with ICC compared to LSIL, HSIL and control groups ($p<0.05$) (Figure 1). Concentrations of IL-1β, TNF-α and IFN-γ were detected, but no differences were observed regarding these cytokine levels among the studied groups ($p\geq0.05$).

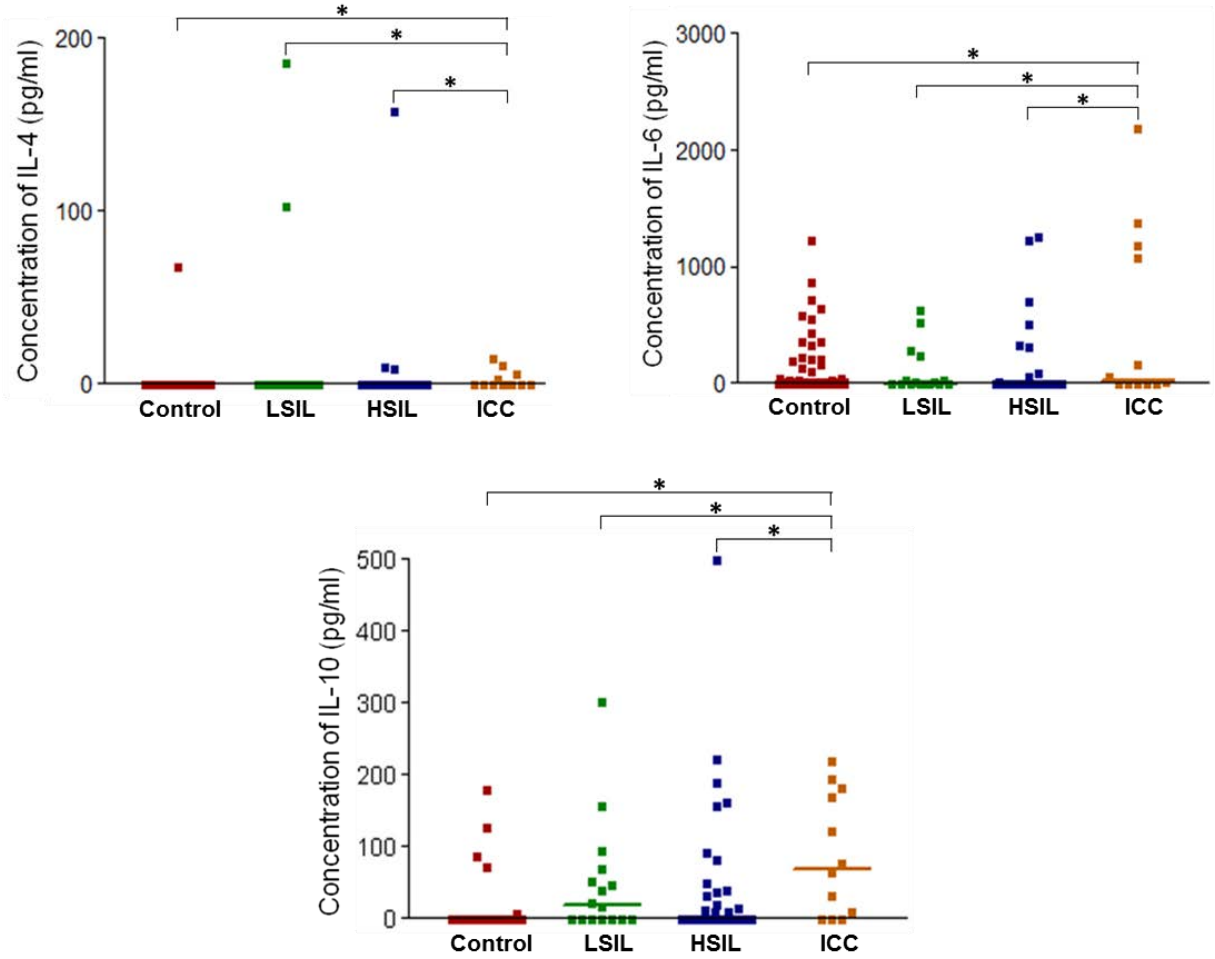

Figure 1. Cytokine concentrations (pg/mL) in cervical secretion samples from the studied patients. *Kruskal-Wallis test ($p<0.05$).

Determination of cytokine concentration in serum

The analysis of IL-6 demonstrated that a significantly higher concentration of this cytokine in patients with HSIL compared to control group, LSIL and ICC ($p<0.05$) (Figure 2). Concentrations of IL-1β, IL-4, IL-10, TNF-α and IFN-γ were detected, but no differences were observed regarding these cytokine levels among the studied groups ($p\geq0.05$).

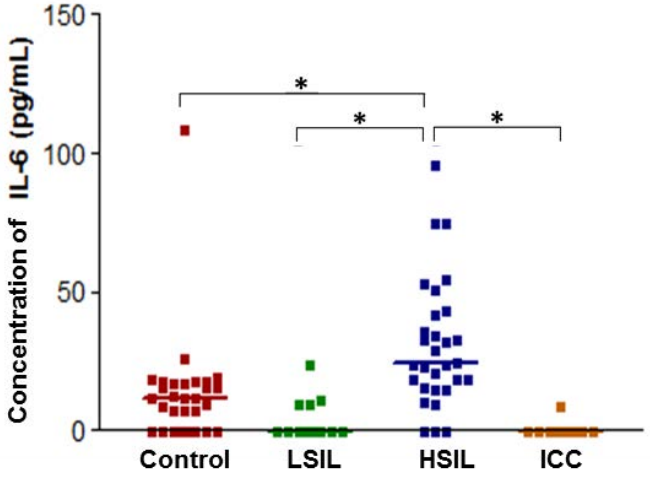

Figure 2. Cytokine concentration (pg/mL) in serum samples from the studied patients. *Kruskal-Wallis test ($p<0.05$).

Determination of immunostaining intensity of cytokines in cervical fragments

The image analysis demonstrated that the mean of INF-γ immunostaining intensity , in pixels^2, was higher in control group compared with LSIL, HSIL and ICC groups, showing statistically significant difference ($p = 0.001$) (Figure 3). In relation to IL-4, the mean of immunostaining intensity, in pixels^2, of this cytokine was higher in HSIL compared to control group ($p = 0.029$) (Figure 4). The means of IL-1β, IL-6, IL-10 and TNF-α immunostaining intensity were similar in all groups, with no statistically significant difference ($p\geq0.05$).

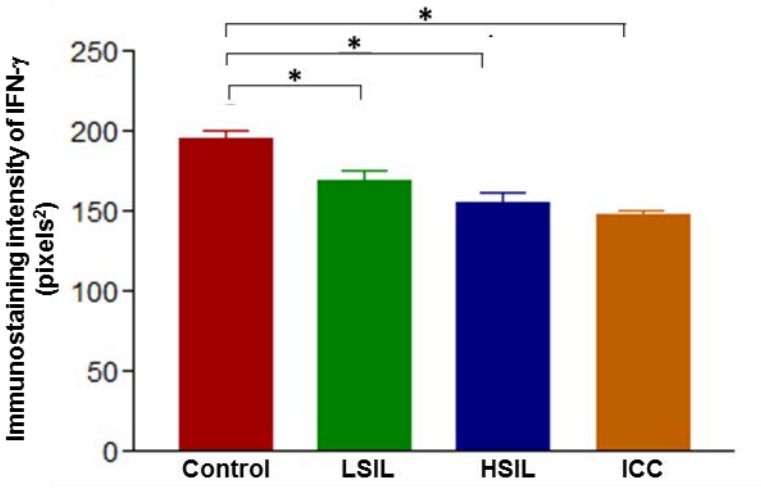

Figure 3. INF-γ immunostaining intensity quantification in the samples included in the study. *Kruskal-Wallis test ($p<0.05$).

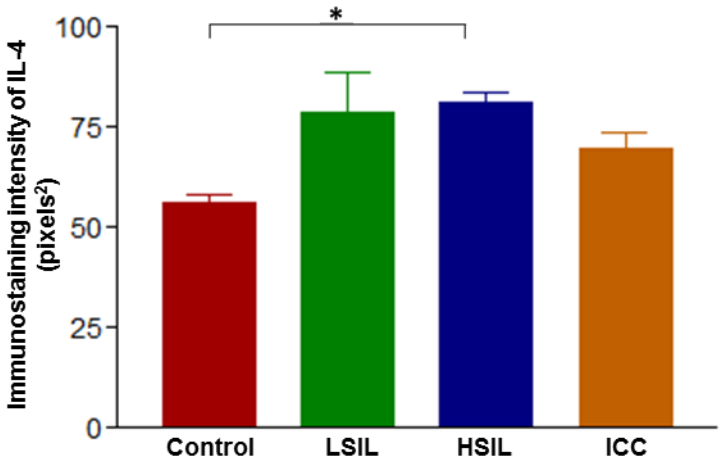

Figure 4. IL-4 immunostaining intensity quantification in the samples included in the study. *Kruskal-Wallis test ($p<0.05$).

Discussion

Epidemiological studies have demonstrated that infection with high-risk HPV types are a significant risk factor for the development of preinvasive cervical lesions and invasive cervical cancer [23, 24]. Recent findings estimate that 10.4% of women worldwide are positive for genital HPV and showed that HPV 16 and 18 were the most frequent genotypes identified in invasive cancers (80.0%). The eight most common HPV types (16, 18, 45, 31, 33, 52, 58 and 35) corresponded to 89.0% of cervical cancers worldwide with different distribution in intraepithelial lesions [25, 26]. These findings are in agreement with our results, in which the positivity for HPV DNA was 93.6% in total samples and HPV 16 was the most prevalent genotype (63.3%) in all groups.

The host immunity, especially in genital tract, is important to combat HPV infections associated with cervical cancers because the local production of cytokines regulates the immune response in different ways. These cytokines modulate the viral replication and polarize the immune response to Th1 or Th2 pattern [27]. Previous studies suggested that the decrease of Th1 and the increase of Th2 immune response are associated with cervical cancer development [27-29]. Clerici et al. [12] reported that the increased secretion of Th2-type cytokines and the decrease of Th1-type ones have been associated with the development of precancerous lesions. Several reports showed that the cervical secretion of patients with HPV infection presented

increased Th2 cytokines [15, 16]. This TH1/Th2 profile can be also modified in the peripheral blood, as suggested by Sharma et al. [13]. These authors observed an increase of IL-4 and IL-10 and a decrease of IL-2 levels in peripheral blood mononuclear cells cultures. In our findings, IL-6 serum concentration was significantly increased in HSIL group, corroborating with studies that reported high levels of IL-6 in sera from patients with intraepithelial neoplasia and cervical cancer [30, 31]. However, IL-4 and IL-10 levels were not different among the groups. These results are consistent with the findings from Mbulaiteye et al. [32], even though they did not find a correlation between plasma Th2 cytokines and HPV detection at the cervix.

Considering the cytokine levels in cervical secretion, it can be observed that Th2 cytokines such as IL-4, IL-6 and IL-10 were significantly higher in patients with invasive cervical carcinoma. However, there was no significant difference in Th1 cytokines among the groups. Our findings are in accordance with studies that demonstrated a correlation between the increased concentration of Th2 cytokines and the severity of the lesion [13, 14, 28, 33]. Mindiola et al. [34] also found an increase of IL-10 production in different lesion degrees suggesting that this might contribute to the mechanism of pre-neoplastic cervical keratinocytes escape, once IL-10 is a potent immunosuppressive cytokine that inhibits activation and the differentiation of Th1 cells [12].

Specific studies on local immune response of cervical cancer are limited, thus, the present study investigated the immunostaining of cytokines in cervical fragments and the association between the Th1/Th2 immune responses and the progression of the lesion. The infiltration of immune cells in cervix tissue lesions that initiates the response to HPV infection leads to the releasing of a variety of immunological mediators. This complex process and the balance of the local immune response may influence on both the infection outcome and the possible progression of preinvasive lesions [35, 36]. Some studies showed a reduction in pro-inflammatory cytokines in cervical carcinoma cells, as well as predominant anti-inflammatory cytokines in precursor lesions, allowing cervical cancer progression [28, 29, 37]. Our findings regarding immunostaining intensity of cytokines in cervical fragments are in agreement with

these studies once we observed an increase of INF-γ immunostaining intensity mean in control group compared with LSIL, HSIL and ICC groups. The secretion of cytokines such as IFN-γ, increase the expression of MHC-I molecules of neoplastic cells and their lysis sensitivity, which potentiates the antitumor response [27]. We also observed an increase of IL-4 in HSIL compared with the control group. According to Olver et al. [38] this cytokine was increased in cervical tissue and vaginal washings in cervical cancer. In addition to the results of immunostaining intensity of Th1 cytokines, we did not find difference in IL-1β and TNF-α expression among the groups. Patients with LSIL and HSIL showed similar expression in TNF-α intensity compared with the control group, suggesting that this cytokine is not associated with lesion severity [37]. In disagreement with our results, studies have shown that there is an association between IL-1 increase and lesion progression [33] which corroborates with the finding that patients with carcinoma presented a significantly elevated expression of pro-inflammatory cytokines [36].

Considering the anti-inflammatory cytokines, there was no IL-6 and IL-10 expression in the evaluated groups. IL-10 is an important regulatory cytokine that plays a role in HPV replication, persistence and malignant transformation and it is highly expressed locally in patients with preinvasive lesions and cervical cancer [39, 40]. Although considered as a Th2-type, some researchers have also reported the production of this cytokine by Th1 cells, which could characterize its antitumor activity [41]. Moreover, IL-10 could be related to the prevention of cervical cancer through HPV clearance [42, 43]. Concerning the IL-6 expression, Mule et al. [44] reported the antitumor activity of this cytokine in a mice model. Depending on its concentration, IL-6 may also exhibits a pro-inflammatory action [45].

Conclusion

Taken together, the results of this study reinforce the increased Th2 cytokines levels and the decreased Th1 ones in premalignant and malignant cervical lesions, which led to the

conclusion that this cytokine profile can induce persistence of HPV infection and promote the cervical lesion progression.

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6. Conclusões

Considerando os resultados obtidos neste estudo a partir do tamanho amostral incluído e das metodologias empregadas, pode-se concluir que:

- A alta frequência de formas integradas de HPV de alto risco oncogênico está associada à severidade das lesões cervicais pré-invasivas, e que a carga viral não parece ser um importante biomarcador para a progressão dessas lesões.
- O aumento dos níveis de citocina de padrão Th2 e diminuição dos níveis de citocina de padrão Th1 nas lesões cervicais pré-invasivas e no carcinoma cervical mostrou que este perfil do padrão de citocinas podem induzir a persistência da infecção pelo HPV e promover a progressão da lesão cervical.

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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Convidamos a senhora para participar da pesquisa intitulada "**Associação de carga viral e estado de integração de HPV e perfil de citocinas em mulheres com neoplasia intraepitelial cervical e câncer cervical"** que tem por objetivo avaliar o papel das proteínas responsáveis pela imunidade, na secreção vaginal e nas biópsias das lesões que podem originar câncer do colo do útero ou nas lesões malignas e associar esse padrão com a quantidade de vírus HPV, que é o vírus associado à essas lesões. Esta pesquisa é de responsabilidade da Mestranda Larissa Doddi Marcolino, que será realizada sob orientação da Profª Drª. Márcia Guimarães da Silva, do Departamento de Patologia da Faculdade de Medicina de Botucatu e participação da Graduanda Danielle Ferreira e Silva do Curso de Ciências Biológicas - Modalidade Médica, da Doutoranda Jossimara Polettini do Programa de Pós-Graduação em Patologia da Faculdade de Medicina de Botucatu, UNESP, do Prof. Dr. João Manuel Grisi Candeias do Departamento de Microbiologia e Imunologia do Instituto de Biociências, da Profa. Adjunta Mariângela Esther Alencar Marques, do Departamento de Patologia da Faculdade de Medicina de Botucatu, UNESP, da Dra. Lenira Maria Queiroz Mauad, da Dra. Ana Lúcia Dalla Déa Trombini e das Enfermeiras Ana Marta B. A. Prado Auler e Célia Regina Chies Gilli do Serviço de Prevenção ao Câncer Ginecológico do Hospital Amaral Carvalho. No momento do exame**,** dentro da rotina estabelecida no Ambulatório, será retirado, um fragmento da lesão que será utilizado para realização dos exames para verificar a presença do vírus HPV, da carga viral e do estado de integração do vírus às células. A pesquisa das proteínas participantes da imunidade será realizada na secreção vaginal, que será colhida, através do exame ginecológico, em que será necessária a introdução de um aparelho de metal, estéril, conhecido como "bico de pato" (espéculo) e também no sangue periférico que será coletado através da punção da veia com seringa estéril, ambos no momento do exame. Os desconfortos desses procedimentos estão restritos à introdução do bico de pato na vagina e ao ardor da punção da veia para retirada do sangue. Além disso, tais procedimentos não precisarão de qualquer tipo de preparo anterior e não trarão nenhum tipo de gasto financeiro para a paciente.

Pelo presente instrumento, eu _____ devidamente esclarecida, ciente da autorização a mim solicitada, não restando quaisquer dúvidas a respeito do lido e explicado, e ciente, também, de que as informações serão utilizadas exclusivamente pelas pesquisadoras, que manterão sigilo sobre minha identidade, e que as mesmas estarão disponíveis para responder a quaisquer perguntas e tornar de meu conhecimento os resultados dos exames e de que posso entrar em contato (por telefone) com as pesquisadoras quando julgar necessário e de que **posso retirar este consentimento a qualquer hora sem prejuízo do meu atendimento neste serviço,** firmo meu **CONSENTIMENTO LIVRE E ESCLARECIDO,** concordando em participar da pesquisa proposta. Estou esclarecida ainda, que não receberei nenhuma gratificação financeira para participar desse estudo a não ser o reembolso de transporte caso tenha que comparecer no hospital somente para participação no estudo.

Botucatu,_____ de _____de 2009

_____

Assinatura da paciente

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em 30 de abril de 1997

Botucatu, 06 de abril de 2009. Of. 124/09-CEP

Ilustríssima Senhora
Profª Drª Márcia Guimarães da Silva
Departamento de Patologia da
Faculdade de Medicina de Botucatu.

Prezada Drª Márcia,

De ordem do Senhor Coordenador deste CEP, informo que o Projeto de Pesquisa, **(Protocolo 3162-2009-CEP)** "Associação de carga viral e estado de integração de HPV e perfil de citocinas em mulheres com neoplasia intraepitelial cervical e câncer cervical", a ser conduzido por Larissa Doddi Marcolino, orientada por Vossa Senhoria, com a participação dos Prof.s Dr.s João Manuel Grissi Candeias, Mariângela Esther Alencar Marques, Paulo Traimam, doutoranda Jossimara Polettini e da graduanda Danielle Ferreira e Silva, recebeu do relator parecer favorável, aprovado em reunião de 06/04/2009.

Ao final da execução deste Projeto, apresentar ao CEP **"Relatório Final de Atividades"**.

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP.

FUNDAÇÃO AMARAL CARVALHO

COMITÊ DE ÉTICA EM PESQUISA
Fundação Hospital Amaral Carvalho

Rua Dr. Miranda Junior, 16, Jardim Alvorada – CEP 17.210-300 – Jaú / SP - ☎ (014) 3602-1194 – Ramal - 1552.

Parecer CEPFHAC – 181/09.

Protocolo de Estudo

Associação de Carga Viral e Estado de Integração de HPV e Perfil de Citocinas em Mulheres com Neoplasia Intraepitelial Cervical e Câncer Cervical

Documentos avaliados:
✓Projeto de Pesquisa Completo.

Pesquisador Responsável:

Autoria: Larissa Doddi Marcolino – Bióloga mestranda em Patologia pela UNESP;
Orientação: Profª. Márcia Guimarães da Silva – Doutora em Patologia - UNESP

O Comitê de Ética em Pesquisa da Fundação Hospital Amaral Carvalho analisou os documentos supracitados na 54ª reunião ordinária realizada no dia 04 de dezembro de 2009. Segue abaixo as considerações feitas pelo relator:

"A pesquisadora responsável apresentou recurso ao Parecer CEPFHAC nº. 145/09, datado de 23 de outubro de 2009, onde encaminha os documentos supramencionados.

O Termo de Consentimento Livre e Esclarecido, apresentado no CEPFHAC no dia 10 de novembro de 2009, traz as cinco restrições sendo estas contempladas: 1 - deixa claro que os exames realizados para coleta de material não trarão qualquer sintoma ou dependerão de alguma preparação que venha acarretar despesas para o participante; 2 – foi informado para evitar constrangimentos futuros que pode haver algum tipo de dor, sensação ou sintoma (incluindo os já conhecidos) durante o procedimento; 3 - o estudo não trará qualquer custo, despesa ou desembolso de valores por parte da participante; 4 - a participante tem o direito de obter acesso aos resultados da pesquisa, bem como o de ligar para a Pesquisadora em caso de qualquer dúvida; e 5 - pode haver reembolso de despesas que sejam decorrentes, exclusivamente, da participação no estudo.

O Questionário de Coleta, apresentado no CEPFHAC no dia 10 de novembro de 2009 não traz qualquer implicação ética para sua não aprovação.

Diante do exposto, sugiro que os documentos aqui analisados sejam aprovados sem restrições."

Diante do exposto, manifestamo-nos pela aprovação sem restrições dos documentos avaliados.

Informamos que os referidos documentos são rubricados pelo colaborador do CEPFHAC, Ricardo Augusto Sartori, e que nenhum dos pesquisadores envolvidos no estudo participou da votação.

Aproveito para recordar-lhe do compromisso de enviar relatórios semestrais referentes à evolução do estudo.

Jaú, 04 de dezembro de 2009.

Éderson R. de Mattos

Dr. Éderson Roberto de Mattos
Coordenador do Comitê de Ética em Pesquisa
Fundação Hospital Amaral Carvalho