MARIA JULLIANA GALVÃO NUNES

EXPRESSÃO DA PROTEÍNA p16 E IDENTIFICAÇÃO DO PAPILOMAVÍRUS HUMANO (HPV) EM LESÕES INTRAEPITELIAIS ANAIS

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Dissertação apresentada ao Programa de Pós-graduação em Patologia do Centro de Ciências da Saúde da Universidade Federal de Pernambuco para obtenção do título de Mestre em Patologia.

Orientador: Prof. Dr. Nicodemos Teles de Pontes Filho
Coorientadora: Profa. Dra. Danyelly Bruneska Gondim Martins

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RESUMO

A infecção pelo HPV (Papilomavírus humano) é o fator de risco mais comum para o desenvolvimento de Neoplasia Intraepitelial Anal (NIA). Além do fator viral deve-se considerar história de intercurso anal, infecção pelo HPV em outros sítios (vulvar, vaginal ou cervical) e tabagismo, bem como processos inflamatórios crônicos anorretais que, associados à imunodepressão, podem acelerar a divisão celular e dar origem à neoplasia. **Objetivo:** Identificar a presença do HPV e avaliar a expressão da proteína p16 em amostras anais de mulheres HIV negativas portadoras de lesões visíveis a anuscopia. **Métodos:** O estudo foi realizado com pacientes atendidas no Hospital de Câncer de Pernambuco, submetidas a anuscopia de magnificação. Diante da presença de lesões entre o canal anal e a zona de transição com o reto, realizava-se a coleta de células anais e biópsia. Foi extraído DNA para posterior análise da identificação do HPV através da Reação em Cadeia Polimerase (PCR), utilizando-se os primers GP5+/6+ e MY09/11 e genotipagem através do PapilloCheck®. Os fragmentos de tecido obtidos por biópsia foram submetidos a rotina histológica, corados pela Hematoxilina-Eosina e a reação imunohistoquímica para identificação da expressão da proteína p16. **Resultados:** Foram classificadas histologicamente 65 amostras e agrupadas como: I-Negativo para displasia: Ia-Normal, alterações benignas e/ou inflamatórias 52,31%, Ib-alterações proliferativas 41,54% e II-Neoplasias 6,15%. Entre os casos negativos para displasia 93.44% não apresentaram marcação para p16. Em 75% dos casos de neoplasias observou-se marcação nuclear e/ou citoplasmática, quanto ao padrão de marcação nesses casos verificou marcação em um terço e três terços do epitélio para NIA I e III, respectivamente. Todas as lesões com diagnóstico histológico de neoplasia apresentaram positividade para HPV para o método da PCR utilizando os primers GP5+/6+ e MY09/11. O primer GP5+/6+ mostrou-se mais eficiente para detecção do DNA-HPV em amostras anais do que o MY09/11. Foram genotipadas 82 amostras e destas 50% apresentaram positividade para 20 genótipos do HPV, sendo HPV6 (18.84%) o mais prevalente seguido por HPV16 (15.94%) e HPV53 (10.14%). Foi observada múltipla infecção em 24.64%, mostrando variação entre 2 – 8 tipos virais. **Conclusões:** O uso de ferramentas adicionais como método molecular (PCR e genotipagem) e imunohistoquímico associados ao exame histológico pode oferecer um diagnóstico do HPV mais preciso nas lesões intraepiteliais anais.

Palavras-chaves: Neoplasia Intraepitelial Anal (NIA), Papilomavírus humano (HPV), proteína p16, Reação em Cadeia Polimerase (PCR) e genotipagem.

ABSTRACT

The infection by HPV (Human Papillomavirus) is the most common risk factor for the development of Anal Intraepithelial Neoplasia (AIN). Besides the viral factor, the history should be considered of anal intercourse, HPV infection at other sites (vulvar vaginal or cervical) and smoking, as well as chronic inflammatory processes anorectal that associated with immunosuppression, can accelerate cell division and lead to cancer. **Objective**: Identify the presence of human (HPV) and evaluate the expression of p16 in anal samples from HIV negative women with lesions visible at anoscopy. **Methods:** The study was conducted with patients attended in service of Pelvis of Cancer Hospital of Pernambuco, submitted to anoscopy magnification verified the presence of lesions between the anal canal and the transition zone with the rectum performed to collect cells and anal biopsy. DNA was extracted for subsequent identification of HPV by Polymerase Chain Reaction (PCR) using the primers GP5+/6+ e MY09/11 and genotyping by PapilloCheck®. The tissue obtained by biopsy was subjected to routine histological sections, stained with hematoxylin-eosin and immunohistochemistry for the identification of p16 protein expression. **Results:** 65 samples were histologically classified and grouped as: Negative for dysplasia (I): 52.31% normal, benign and/or inflammatory (Ia), 41.54% proliferative changes (Ib) and 6.15% Neoplasias (II). Between the cases negative for dysplasia 93.44% did not show marking for p16. In 75% of the cases of neoplasia observed nuclear staining and/or cytoplasmatic, as the standard of marking in these cases observed in one third and three third of the epithelium to NIA I and III, respectively. All lesions with a histological diagnosis of neoplasia were positive for HPV by PCR method using the primers GP5+/6+ e MY09/11. The primer GP5+/6+ were more efficient for detection of HPV DNA in anal samples than the MY09/11. 82 samples were genotyped and of these 50% showed positive for HPV genotypes 20, being HPV6 (18.84%) the most prevalent, followed by HPV16 (15.94%) and HPV53 (10.14%). Multiple infections were observed in 24.64%, showing variation between 2-8 virus types. **Conclusions**: The use of additional tools like molecular method (PCR and genotyping) and immunohistochemical associated with histological examination can provide a more accurate diagnosis of NIA.

Key-words: Anal Intraepithelial Neoplasia (AIN), human papillomavirus (HPV), p16 protein, Polymerase Chain Reaction (PCR) and genotyping.

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LISTA DE ABREVIATURAS/SIGLAS

CCS – Centro de Ciências da Saúde

CDK - quinase dependente de ciclina

DAB - Diaminobenzidina

DNA – Ácido desoxiribonucleico

E - gene que codifica proteína imediata ou *early* do Papilomavirus humano

E2F - família de fatores de transcrição específica

G1 - período de crescimento celular

HCP – Hospital de Câncer de Pernambuco

HIV – Human immunodeficiency virus

HPV – Human papillomavirus

INCA – Instituto Nacional do Câncer

L - gene que codifica proteína tardia ou *Late* do Papilomavirus humano

LCR - longa região de controle

LIKA – Laboratório de Imunopatologia Keizo Asami

NIA – Neoplasia Intraepitelial Anal

PCR - Reação em cadeia da polimerase

pRb – proteína do retinoblastoma

p16 – proteína 16

p53 - proteína 53, supressora de tumor

S - período de síntese (replicação) celular

UFPE – Universidade Federal de Pernambuco

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1. APRESENTAÇÃO

De acordo com levantamento do INCA cerca de 1.617 novos casos de câncer anal foram diagnosticados em 2009, sendo 539 em homens e 1.078 em mulheres, revelando uma maior freqüência nestas últimas (INCA, 2009). Entre os homens, no entanto a incidência do câncer anal é elevada em homossexuais (35 casos por 100.000 habitantes), sendo mais alarmante na população com síndrome da imunodeficiência adquirida (SIDA), porém, as lesões intraepiteliais anais também têm sido encontradas tanto em pacientes masculinos homossexuais quanto em mulheres HIV-negativos (CHANG *et al*., 2002; Mc CLOSKEY *et al.*, 2007).

Os tumores anais ocorrem na borda ou no canal anal, indo até a transição com o reto. Os tumores que surgem na borda anal são lesões dermatológicas e, como tal, podem ser tratados apenas com excisão local. Por outro lado, aqueles presentes no canal anal ou na zona de transição com o reto exigem tratamentos mais agressivos (RYAN; COMPTON; MAYER, 2000).

O câncer anal é sintomático apresentando dor anal, irritação, sangramento e sensação de massa retal, porém cerca de 20% dos pacientes não apresentam sintomas até o momento do diagnóstico (ELIASHVILI; LANDRY; MATTHEWS, 2002). O tipo histológico mais comum, o carcinoma de células escamosas, surge em áreas de NIA de alto grau, como conseqüência de infecção crônica pelo Papilomavirus humano (HPV) e parece ter relação com alta carga do vírus na infecção (FOX, 2006). As lesões por ele provocadas são classificadas como neoplasias intraepiteliais graus I (leve), II (moderada) e III (grave) e podem estar associadas aos subtipos do HPV infectante. De acordo com a sua localização, são referidas com as seguintes abreviaturas: Neoplasia Intraepitelial Anal (NIA) e neoplasia intraepitelial perianal (NIPA) (CAPOBIANGO, 2008).

A infecção pelo HPV é o fator de risco mais comum para o desenvolvimento de NIA (GARRETT; KALADY, 2010). Outras condições incluem história de intercurso anal, histórico de HPV em outros sítios (vulvar, vaginal ou cervical) e tabagismo (DALING *et al*., 2004; McCLOSKEY *et al*., 2007; PALEFSKY, 2010). Deve ser levado em conta, entretanto, que a associação entre a infecção por HPV e o aparecimento de tumores anais acontece em um contexto mais amplo do que a simples presença do vírus. Assim, além do fator viral, são importantes os processos inflamatórios crônicos anorretais que, associados à imunodepressão, podem acelerar a divisão celular e dar origem à neoplasia (NADAL; MANZIONE, 2004).

O HPV apresenta tropismo pela pele e membranas mucosas. O canal anal, assim como todo o trato genital, faz parte do circuito viral do HPV ou até mesmo pode ser um reservatório para o mesmo, devido a presença de epitélio escamoso (DALING *et al*., 2004; PALEFSKY,

2010). Atualmente, sabe-se que o vírus pode ser encontrado também em câncer de pênis, vagina, vulva, ânus, região perianal, cabeça e pescoço. Na região anogenital pode causar verrugas genitais e levar ao desenvolvimento de lesões com possibilidades de progressão para o câncer (HAUSEN, 2009; ESQUENAZI *et al*., 2010).

O HPV, vírus de DNA, pertence à família *Papillomaviridae*, é caracterizado por apresentar um capsídeo icosaédrico com um diâmetro de 50nm a 60nm, não revestido por um envelope lipídico, possuindo 72 capsômeros e determinantes antigênicas espécies-específicas na superfície externa e interna (ESQUENAZI *et al*., 2010). Seu genoma é constituído por 8.000 pares de bases e pode ser dividido em três regiões: a região precoce (*early*) contendo os genes E1, E2, E4, E5, E6 e E7 que são necessários à replicação viral e com propriedades de transformação oncogênica; região tardia (*late*) contendo os genes L1 e L2 que possuem códigos para a formação de proteínas do capsídeo viral e a região regulatória (LCR) com genes que controlam a replicação e os elementos para transcrição (ALBRING; BRENTANO; VARGAS, 2006).

A infecção pelo HPV ocorre quando o vírus se instala nas células da camada basal do epitélio escamoso através de microtraumas. Na camada basal a replicação é não-produtiva, ou seja, é mínima, não se produzindo alterações identificáveis histologicamente como, coilocitose e atipias celulares. Já nas células escamosas diferenciadas das camadas suprabasais ocorre uma replicação do tipo produtiva com evidentes manifestações morfológicas (ELEUTÉRIO JÚNIOR; GIRALDO; GONÇALVES, 2006).

O DNA viral dentro da célula do hospedeiro pode assumir duas formas, a episssomal e a integrada. Na forma episssomal, o DNA viral permanece circular no núcleo da célula do hospedeiro, não estando integrado ao DNA da mesma. Essa forma é encontrada nas verrugas genitais e lesões de menor gravidade. Para a integração do genoma circular ao DNA da célula hospedeira, esse deve ser linearizado, pela quebra do DNA viral entre a região E1 e L1, resultando na ruptura ou perda do gene E2, sendo encontrado nas lesões de maior gravidade, como o carcinoma "*in situ*" e invasivo (ALBRING; BRENTANO; VARGAS, 2006).

A transformação maligna induzida pelo HPV depende da integração dos genes E6 e E7 do HPV oncogênico ao genoma do hospedeiro, à persistência da infecção, à baixa de imunidade celular e conseqüente expressão das proteínas E6 e E7. Essas proteínas, produtos de oncogenes virais, são fundamentais para garantir as características de malignidade do HPV. A oncoproteína viral E6 liga-se a p53, proteína supressora tumoral e a degrada por ubiquitinação proteossômica, levando à resistência à apoptose. Já a E7 interage com a pRb, que serve como um freio que limita a entrada na fase S, ao ligar-se às proteínas E2F,

reguladoras de genes. A E7 é uma potente inibidora da atividade da p21 e p27, inibidores de quinase ciclina-dependentes (CdK), evitando o controle de checagem da fase G1. A proteína p16 atua como inibidora de CdK, mantendo a pRb hipofosforilada e controlando negativamente a progressão do ciclo celular (JONES *et al*., 1997; TOKUGAWA *et al*., 2002; TRINGLER *et al*., 2004).

Quando o HPV se integra ao genoma da célula hospedeira, há um rompimento do genoma viral. Com isso, há perda da função da proteína E2, que é a proteína regulatória das oncoproteínas virais, E6 e E7. Subsequentemente ocorre ativação da região promotora de genes envolvidos na síntese de DNA e na progressão do ciclo celular, resultando na superexpressão da p16, proteína reguladora negativa do ciclo celular (TRINGLER *et al.*, 2004).

A proteína p16 apresenta expressão claramente associada ao mecanismo transformante viral, desta forma pode auxiliar no diagnóstico de lesões intraepiteliais escamosas de alto grau, por vezes, morfologicamente difíceis de serem diferenciadas de quadros reparativos intensos e de lesões de menor grau com importantes alterações reativas, bem como na identificação de lesões com maior potencial evolutivo para a invasão (KLAES *et al*., 2002; WANG *et al*., 2004; O'NEILL; McCLUGGAGE, 2006).

A principal estratégia na prevenção e no controle das neoplasias malignas é sua detecção precoce, permitindo que intervenções e terapias efetivas possam contribuir para a redução da mortalidade e morbidade por câncer.

Este trabalho teve como objetivo geral identificar a presença do HPV e avaliar a expressão da proteína p16 em amostras anais de mulheres HIV negativo portadoras de lesões anais visíveis a anuscopia de magnificação. Os objetivos específicos deste estudo foram: verificar a expressão de p16 em epitélio anal através do método imunohistoquímico; detectar DNA do HPV nas amostras citológicas anais; correlacionar os níveis de expressão da proteína p16 com os resultados dos exames histopatológicos e correlacionar o DNA do HPV com os resultados dos exames histopatológicos.

O desenvolvimento dessa dissertação resultou na elaboração de três artigos. O primeiro, um artigo de revisão sistemática intitulado "Correlação da expressão da proteína $p16^{INK4a}$ com o grau de Neoplasia Intraepitelial Anal: Uma revisão sistemática", publicado na Revista Paraense de Medicina. O segundo artigo intitulado "p16 protein expression in identification of anal intraepithelial lesions related to Human papillomavirus (HPV)" submetido para publicação no *International Journal of Infectious Diseases*. O terceiro artigo

intitulado “Identification and genotyping of Human papillomavirus in anal samples of women HIV-negatives” submetido para publicação no *Journal of Clinical Virology*.

2. REVISÃO DE LITERATURA

ATUALIZAÇÃO/REVISÃO

CORRELAÇÃO ENTRE A EXPRESSÃO DA PROTEÍNA p16^{INK4a} E O GRAU DE NEOPLASIA INTRA-EPITELIAL ANAL: UMA REVISÃO SISTEMÁTICA[1]

CORRELATION BETWEEN EXPRESSION OF p16I^{NK4A} PROTEIN AND DEGREE OF ANAL INTRAEPITHELIAL NEOPLASIA (AIN): A SYSTEMATIC REVIEW

Maria Julliana Galvão NUNES[2], Guacyra Magalhães PIRES[2], Darley de Lima Ferreira FILHO[2], Adrya Lúcia Peres Bezerra de MEDEIROS[3] e Nicodemos Teles de PONTES-FILHO[4]

RESUMO

Objetivo: identificar pela revisão sistemática a expressão da proteína p16^{INK4a} nos diferentes graus de neoplasias intra-epiteliais anais (NIA). **Método:** revisão sistemática de pesquisa nas bases de dados: PubMed, MedLine Old, MedLine, LILACS, SciELO e Science Direct. Foram excluídos artigos de revisão e que não correlacionavam a expressão da p16^{INK4a} com o grau de NIA. **Resultados:** foram encontrados 483 artigos, dos quais 223 na PubMed, 151 na Medline, 34 na Medline Old, 3 no Lilacs e 72 na Science Direct. Apenas cinco artigos foram selecionados baseados no critério de inclusão e exclusão. **Conclusão:** a proteína p16^{INK4a} utilizada como marcador mostra-se eficaz para o diagnóstico de NIA, principalmente em lesões anais de alto grau.

Descritores: imunoistoquímica, p16^{INK4a}, neoplasia intra-epitelial anal, revisão sistemática.

INTRODUÇÃO

Há uma crescente incidência de câncer anal em países ocidentais, no entanto os dados são limitados quanto a sua causa primária, a infecção do canal anal pelo Papillomavirus humano (HPV)[1]. As lesões intra-epiteliais do canal anal têm sido encontradas em número elevado nos pacientes masculinos homossexuais HIV (Vírus da Imunodeficiência Adquirida) negativo[2] e em mulheres HIV negativo, cuja explicação pode estar relacionada a três fatores: infecção por vírus dos subtipos oncogênicos, maior número de coitos anais que o relatado e contaminação oriunda de infecção genital[3,4]. Esta incidência atinge proporções epidêmicas nos homossexuais masculinos (35 casos por 100.000 habitantes), sendo ainda mais alarmante na população com síndrome da imunodeficiência adquirida (SIDA)[5].

O tipo histológico mais comum do câncer anal, o carcinoma de células escamosas (CCE), surge em áreas de neoplasias intra-epiteliais anais (NIA) de alto grau, como conseqüência de infecção crônica pelo HPV e parece ter relação com alta carga do vírus na infecção[6].

A transformação maligna no canal anal depende da integração dos genes E6 e E7 do HPV oncogênico ao genoma do hospedeiro, à persistência da infecção, à baixa de imunidade celular e conseqüente expressão das proteínas E6 e E7. Essas proteínas são produtos de oncogenes virais capazes de interagir com proteínas controladoras do ciclo celular, p53 e pRb[7,8]. Em decorrência dessa interação é ativado um processo de retroalimentação negativo que resulta na expressão da proteína inibitória dos complexos de quinases dependentes de ciclina, a p16^{INK4a} [9], e a célula é conduzida à transformação, imortalização e,

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posteriormente, ao câncer[7].

A imunomarcação da proteína p16^{INK4a} é uma ferramenta eficaz na rotina laboratorial da patologia anal contribuindo para uma melhor precisão do diagnóstico de NIA, especialmente em casos limítrofe[10]. Poucas medidas na Medicina diagnóstica tiveram tanto impacto quanto a introdução, nas práticas de laboratório clínico, dos imunoensaios para a determinação de proteínas relacionadas a tumores, também classificadas como marcadores tumorais[11].

OBJETIVO

Realizar uma revisão sistemática sobre a expressão da proteína p16INK4a nos diferentes graus de neoplasias intra-epiteliais anais (NIA).

MÉTODO

Revisão sistemática realizada no período de setembro de 2010 a outubro de 2010, a partir das bases de dados online: PUBMED (U. S. National Library of Medicine), MEDLINE OLD, (Literatura Internacional em Ciências da Saúde – até 1996), MEDLINE (Literatura Internacional em Ciências da Saúde, a partir de 1997), LILACS (Literatura Latino-Americana e do Caribe em Ciências da Saúde), SciELO (Scientific Electronic Library Online) e SCIENCE DIRECT, utilizando-se como descritores (DeCs): imunoistoquímica e neoplasias de ânus, e como termos livres (TL): biomarcadores, p16^{INK4a}, neoplasia intra-epitelial anal, todos com seus correspondentes em inglês. Estes foram cruzados nas bases de dados da seguinte forma: imunoistoquímica e neoplasias de ânus; biomarcadores e neoplasia intra-epitelial anal e p16^{INK4a} e neoplasias de ânus. Foram incluídos artigos originais nos idiomas: português, inglês e espanhol. Excluídos artigos de revisão, monografias, dissertações e livros, artigos que não correlacionavam a expressão da p16^{INK4a} com o grau de NIA, que utilizavam como método a imunocitoquímica e aqueles nos quais os pacientes haviam recebido tratamento prévio. Foram também pesquisadas as referências dos artigos incluídos. Não houve limitação do período de publicação dos artigos pesquisados.

RESULTADOS

Inicialmente foram encontrados 483 artigos, dos quais 223 foram na PubMed, 151 na Medline, 34 na Medline Old, 3 no Lilacs e 72 na Science Direct. Foram excluídos 472 artigos por não terem relação com o tema ou serem duplicatas. Após a leitura do artigo original foram selecionados cinco artigos atendendo aos critérios pré-estabelecidos.

Optou-se por considerar as seguintes variáveis nos artigos selecionados: autor/ano, amostragem, métodos, resultados e conclusões (Tabela I).

DISCUSSÃO

A detecção de alterações celulares que podem ser originadas pela expressão desregulada das oncoproteínas virais pode caracterizar marcadores envolvidos na progressão tumoral e, desta forma, contribuir para a identificação de grupos de células que apresentem maior risco de progressão para o câncer. Na neoplasia associada ao HPV a desregulação da expressão do oncogene viral E7 interfere na regulação da rede de ciclinas celulares, levando a superexpressão da proteína p16^{INK4a} [16], uma proteína inibitória dos complexos de quinases dependentes de ciclina[9], que regula a transição da fase G1 para a fase S do ciclo celular[16].

TABELA I – Estudos que analisaram a expressão da p16INK4a em Neoplasia Intra-epitelial Anal (NIA)

AUTOR/ANO	AMOSTRAGEM	MÉTODOS	RESULTADOS	CONCLUSÕES
Bean et al, 2009[12]	77 biópsias anal	Diagnóstico H&E IHC: Coloração nuclear e/ou citoplasmática para $p16^{INK4a}$ < 5% - negativa. Lesões NIA I/HPV: reatividade para p16 irregular ou descontinua 1/3 basal do epitélio escamoso. Lesões de alto grau: reatividade difusa ou continua na camada basal/parabasal nos 2/3 superiores ou mais.	Casos: 25 normal/reativa, 28 NIA I, 3 NIA II e 13 NIA III	Acredita-se que p16INK4a é melhor usado para casos desafiadores, onde o diagnóstico diferencial situa-se entre a metaplasia escamosa atípica e NIA
Kreuter et al, 2009[10]	49 espécimes de mucosa anal normal e displasia de alto e baixo grau	NIA graus histológicos: 1/3 inferior- NIA I, menor 2/3- NIA II ou todo epitélio - NIAIII. NIA I baixo grau e NIA II e III de alto grau. IHC: Coloração nuclear com ou sem coloração citoplasmática - positiva para $p16^{INK4a}$. Padrões de coloração: irregular (>10% das células escamosas espalhadas marcadas); Banda (>90% contíguo de células escamosas marcadas).	Mucosa anal normal - imunocoloração ausente ou apenas positiva em poucas células isoladas. NIA de baixo grau - expressão irregular ou em 5% ou menos das células. NIAs de alto grau - positivas para $p16^{INK4a}$.70,8% - padrão de banda e expressão entre 55% a 86% das células epiteliais.	$p16^{INK4a}$ como auxiliar no diagnóstico de NIA de alto grau em homossexuais masculinos HIV-positivo. Pode ser usada em indivíduos HIV negativos com displasia associada ao HPV.
Bernard et al, 2008[13]	108 biópsias anal consecutivas e 25 espécimes consecutivas de hemorroidectomia	NIA I, II, ou III de acordo com grau de displasia. IHC: intensidade de $p16^{INK4a}$ nuclear: 1+ (intensidade fraca) e 2+ e 3+ (intensidade forte). Padrão nuclear foi incluído: raro, desigual, contíguo, basal, basal-superficie e superficie.	100% de NIA II e III - fortemente positivos (2 ou 3+) para $p16^{INK4a}$ nuclear e 80% para NIA I. 98% dos casos de NIA III, 100% de NIA II e 28% dos NIA I - padrão de coloração contíguo - 100% NIA III /II e 48% NIA I forte intensidade nuclear. 33% NIA negativos foram positivos para $p16^{INK4a}$ com menor intensidade do que para NIA II /III.	A utilidade do diagnóstico da IHC para p16INK4a é maior em NIA II e III e auxilia no dilema do diagnóstico de NIA de alto grau versus zona de transição do epitélio benigno.
Bean et al, 2007[14]	75 espécimes/ 55 pacientes	Grau de displasia: menos de 1/3- NIA I; 2/3 – NIA II; carcinoma *in situ* com células displásicas ocupando mais de 1/3 – NIA III. IHC: Padrão da coloração de $p16^{INK4a}$; negativo, descontínuo (focal), ou difuso/contínuo. Positiva: presença de reatividade nuclear e/ou citoplasmática. Negativa: coloracão em < 5% das células.	47% dos casos - negativo para displasia; 31% - displasia de baixo grau; 23% - displasia de alto grau. Coloração nuclear e/ou citoplasmática observada na maioria (74%) dos casos de NIAs de baixo e alto grau. NIA de baixo grau: coloração focal ou desigual NIA de alto grau: difusa.	Em NIA de alto grau o padrão de coloração para p16 tende a ser de forma difusa. A p16 mostrou-se um marcador sensível e confiável para diagnosticar NIA de alto grau.
Walts et al, 2006[15]	104 biópsias anal de 74 pacientes e biópsia cervical positiva	Casos: 37 negativos, 12 condilomas, 14 NIA I (displasia leve), 25 NIA II (displasia moderada) e 16 NIA III (grave displasia / CIS). IHC: Coloração nuclear e/ou nuclear e citoplasmática em >10% das células escamosas - positivo; Apenas citoplasmática - inespecifica negativa. Padrões de coloração: em banda (>90% das células escamosas contiguas foram coradas) ou manchada (>10% das células com coloração positiva espalhada).	83% de NIA de alto grau (NIA II e III) e 21% de NIA I – padrão de banda. Padrão de banda nos casos de NIA I foram nível 1. Padrão em manchas: células positivas distribuídas desigualmente em vários níveis do epitélio e não correlacionaram com o diagnóstico. Em todos os casos NIA de alto grau com padrão de banda, > 25% das células escamosas coraram positivas.	p16 é um complemento muito útil a morfologia no diagnóstico histológico na gradação de NIA associada ao HPV, pode melhorar a precisão do diagnóstico patológico e deve ser um componente na avaliação de biópsias que são difíceis de avaliar NIA apenas pela morfologia.

(Abreviaturas: IHC: Imunoistoquímica; NIA: Neoplasia Intra-epitelial Anal; HPV: Papillomavirus humano; HIV: Vírus da Imunodeficiência Adquirida)

Observando o ano de publicação dos artigos selecionados, nota-se que estes são bastante recentes, afirmando que a expressão da proteína em amostras anais foi pouco estudada em décadas anteriores. A maioria dos estudos que verificam a expressão da proteína p16^{INK4a} utilizam amostras de colo uterino9, o que pode justificar o baixo número de artigos encontrados relacionados ao tema.

Nos cinco artigos selecionados a maioria dos estudos utilizaram número da amostra semelhantes, com exceção do estudo de Kreuter et al[10] que utilizou uma amostragem menor. Apenas um estudo[15] utilizou biópsia cervical positiva como controle. Alguns estudos[12-14] avaliaram amostras provenientes de hemorroidectomia ou casos de alterações de células escamosas reativas como padrão negativo para reatividade da proteína p16^{INK4a}, visto que esta proteína não é expressa em epitélio normal, células proliferativas e lesões inflamatórias.

Estudo utilizando a proteína p16^{INK4a} como marcador mostra um resultado com 75% de sensibilidade e 100% de especificidade no diagnóstico de NIA[15]. Alguns estudos que correlacionam a expressão desta proteína com grau de NIA apontam esta proteína como um biomarcador eficaz principalmente para diagnosticar NIA de alto grau[13,14]. Kreuter et al10, afirmam que a expressão da proteína p16INK4a não é, significativamente, diferente entre a mucosa anal normal e aquela com NIA de baixo grau. Estes dados são concordantes com os de outros autores que analisando casos negativos de NIA encontraram positividade para p16INK4a, embora em menor intensidade quando comparados aos de NIA positivos[14].

Também foi relatado a superexpressão de p16^{INK4a} em casos de carcinoma de células escamosas[17]. De acordo com Walts et al[15], a superexpressão da p16^{INK4a} em lesões intra-epiteliais anais, cervicais e vulvar associadas ao HPV e carcinomas pode ser conseqüência da integração do DNA do HPV de alto risco a célula hospedeira.

Um estudo analisando homossexuais masculinos HIV positivos, grupo que apresenta alto risco para o desenvolvimento de displasia anal, revelou que a p16^{INK4a} auxilia no diagnóstico de NIA de alto grau, indicando a proteína como biomarcador adicional efetivo na rotina da patologia anal, melhorando a precisão no diagnóstico de NIA[10].

Em relação ao padrão de coloração encontrado nos casos de NIA, os autores revelam um padrão em banda ou difuso com maior intensidade para os casos de NIA de alto grau (NIA II e III), já nos casos de NIA de baixo grau (NIA I) a marcação apresenta-se como focal ou desigual e, mesmo quando mostra padrão em banda, esta é pouco intensa[10,14,15]. As lesões anais de alto grau, geralmente, apresentam reatividade para p16^{INK4a} de forma contínua, ao contrário das lesões de baixo grau. Isso se deve ao fato de que um maior número de células nas lesões de alto grau estão envolvidas no processo de malignidade e conseqüentemente são marcadas mais intensamente pela proteína.

Observou-se que na maioria dos artigos analisados[10,12,14,15] foi verificado o grau histológico de NIA. Nestes não se pôde verificar uma concordância quanto ao número de casos de NIA de baixo grau e NIA de alto grau, pois nos estudos de Bean et al[12] e Bean et al[14] foi observado um maior número de casos de NIA de baixo grau (NIA I), no entanto, pode-se levar em consideração que ambos estudos fazem parte do mesmo grupo de pesquisa e ambos divergem dos estudos de Kreuter et al[10] e Walts et al[15] que mostram um número de casos de NIA de alto grau (NIA II e III) consideravelmente maiores que os casos de NIA de baixo grau.

CONSIDERAÇÕES FINAIS

Conforme revisão sistemática, a proteína p16^{INK4a} utilizada como marcador mostra-se eficaz para o diagnóstico de NIA, principalmente, em lesões anais de alto grau.

SUMMARY

CORRELATION BETWEEN EXPRESSION OF p16INK4A PROTEIN AND DEGREE OF ANAL INTRAEPITHELIAL NEOPLASIA (AIN): A SYSTEMATIC REVIEW

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Objective: to identify through a systematic review the expression of $p16^{INK4a}$ protein in different degrees of anal intraepithelial neoplasia (AIN). **Method:** the review was realized through research on databases PubMed, MedLine Old, MedLine, LILACS, SciELO e Science Direct. Review articles and the ones without correlation between the expression the $p16^{INK4a}$ with the level of AIN were excluded. **Results:** it were found 483 articles, which 223 in PubMed, 151 in Medline, 34 in Medline Old, 3 in Lilacs and 72 in Science Direct. Only five articles were selected based on inclusion and exclusion criteria. **Conclusion:** the $p16^{INK4a}$ protein used as a marker has shown to be effective for diagnostic of AIN, mainly in high-grade anal lesions.

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3. MÉTODOS

3.1 Área

A coleta de material desta pesquisa foi realizada no Serviço de Pélvis do HCP. O processamento das amostras e todos os procedimentos foram realizados no Setor de Patologia do HCP e do LIKA, na UFPE.

3.2 População

A pesquisa foi realizada com pacientes voluntárias atendidas no Serviço de Pélvis do HCP, no período de Março a Outubro de 2011.

3.3 Seleção da Amostra

3.3.1 Critérios de inclusão

Pacientes com idade entre 18 e 60 anos, HIV negativo e com indicação para o exame anuscopia de magnificação foram incluídas neste estudo. Para indicação do exame as pacientes obedeceram aos seguintes critérios: histórico de lesões provocadas pelo HPV na região anogenital e queixa de prurido e/ou dor na região anal.

3.3.2 Critérios de exclusão

Foram excluídas deste estudo pacientes com ausência de lesão entre o canal anal e a zona de transição com o reto, visível a anuscopia de magnificação, menores de 18 anos e maiores de 60 anos, HIV positivo, em tratamento para regressão de lesões na região anal no período de execução da pesquisa.

3.4 Método de Coleta

3.4.1 Obtenção da amostra

Para obtenção das amostras, inicialmente foi realizada a anuscopia de magnificação utilizando-se anuscópio e colposcópio e aplicação de ácido acético a 5% para visualização de possíveis lesões no canal anal e zona de transição entre o canal anal e o reto. Diante da presença de lesões foram coletadas amostras biológicas com o auxílio de escova ginecológica introduzida no canal anal. As escovas foram colocadas em tubos contendo 1ml de soro fisiológico.

Em seguida foi realizada anestesia local e biópsia da área lesionada, sendo os fragmentos fixados em formalina a 10% tamponada, incubados por um período mínimo de 48 horas e emblocados em parafina.

3.5 Procedimento histológico e imunohistoquímico

O exame histopatológico foi realizado no Setor de Patologia do HCP. A partir dos blocos obtidos foram retirados cortes histológicos com 4μm de espessura, que posteriormente foram corados com Hematoxilina-Eosina (HE) para análise morfológica.

Os cortes para realização do procedimento imunohistoquímico foram obtidos no setor de Patologia do HCP, o processamento foi realizado no Setor de Patologia do LIKA, utilizando-se o kit CINtec® Histology para a detecção em tecido da $p16^{INK4a}$. Os cortes histológicos foram montados em lâminas silanizadas, desparafinizados e incubados em solução de recuperação antigênica (1:10), previamente aquecida em *steamer* com temperatura entre 95 – 99°C, por cerca de 10 minutos. Após resfriamento em tempetura ambiente as lâminas foram lavadas com tampão de lavagem (1:10). Em seguida adicionou-se 50μl do reagente para bloqueio da peroxidase endógena (3% peróxido de hidrogênio e azida sódica-NaN_3) sobre o tecido, e incubou por 5 minutos em temperatura ambiente. Após o período de incubação foi repetida a lavagem.

As lâminas foram retiradas do tampão de lavagem, secas ao redor do corte, e adicionou-se 50µL do anticorpo monoclonal primário (clone E6H4) anti-p16 e incubou-se por 30 minutos em temperatura ambiente e, posteriormente, lavadas com tampão de lavagem.

Após lavadas e secas, adicionou-se 50µL do reagente de visualização, e foram incubadas por 30 minutos, em temperatura ambiente. A lavagem foi repetida por duas vezes, e após serem secas foi aplicado 50µL do cromógeno DAB (3, 3' -diaminobenzidina), incubou-se por 10 minutos em temperatura ambiente e repetindo-se a lavagem. Foi realizada a contra-coloração com Hematoxilina de Harris e montagem das lâminas com Entellan® e lamínulas. A visualização da marcação foi realizada utilizando-se microscópio de luz convencional.

3.6 Extração de DNA

O procedimento foi realizado no Setor de Biologia Molecular no LIKA/UFPE. Para extração de DNA das amostras anais contidas nas escovas ginecológicas foi utilizado o kit DNA IQ™ Casework Pro (Promega®) utilizado no equipamento para extração automatizada Maxwell® 16, seguindo instruções do fabricante. Ao final do processo as amostras foram armazenadas a uma temperatura de -20ºC para posterior realização dos testes de amplificação da sequência viral.

Para garantir a qualidade da extração do DNA das amostras biológicas foi utilizado o gene repórter β-globina, utilizado para amplificar uma região de 268pb. As condições da PCR foram desnaturação inicial por 10 minutos a 94°C, seguido de 40 ciclos de desnaturação a 94°C por 1 minuto, anelamento a 62°C por 1 minuto e extensão a 72°C por 1 minuto. A etapa de extensão final foi realizada a 72°C por 7 min.

3.7 Amplificação do genoma e identificação do HPV

A presença do HPV nas amostras biológicas foi realizada no Setor de Biologia Molecular no LIKA/UFPE através da técnica de Reação em Cadeia Polimerase - PCR, utilizando-se os primers consensus GP5+/6+ e MY09/11, com fragmentos de 150 pb e 450pb, respectivamente. O volume final da reação foi de 12,5 µL contendo 1 µL do DNA extraído,

1uL de cada primer (numa concentração de 10pMol), 3,25uL de água ultrapura e 6,25uL de GoTaqGreen Master Mix (Promega®). As condições de amplificação foram: (i) 94°C por 3 minutos; (ii) 34 ciclos de desnaturação a 95°C por 1 minuto, anelamento por 1 minuto e extensão a 72°C por 1 minuto; (iii) extensão final a 72°C por 10 min. Os primers consensus para a região do gene L1 GP5+/6+ (5'-TTTGTTACTGTGGTAGATACTAC-3'/5'-GAAAAATAAACTGTAAATCATATTC-3') com temperatura de anelamento de 45°C e os primers MY09/11 (5'-CGTCCMARRGGAWACTGAT-3'/5'-GCMCAGGGWCATAAYAATGG-3') com temperatura de anelamento de 55ºC. Como controle positivo foi utilizado o plasmídio pBR322.HPV16, gentilmente cedido pelo Instituto Butantan, e como controle negativo da reação foi utilizado água ultrapura. O produto da PCR foi visualizado em gel de agarose 1% com brometo de etídio (10mg/ml) sob luz ultravioleta.

As amostras foram genotipadas utilizando o PapilloCheck® HPV-screen DNA-chip (Greiner Bio-one®), seguindo instruções do fabricante. É possível identificar 24 genótipos do HPV, sendo 06 de baixo risco (06, 11, 40, 42, 43, 44/55) e 18 de alto risco (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82). O PapilloCheck® é baseado na amplificação por PCR do gene E1 do HPV.

3.9 Comitê de Ética

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, sob processo CPE/CCS/UFPE Nº280/10 (ANEXO A).

4. RESULTADOS

4.1 Artigo Original 1

REVISTA: International Journal of Infectious Diseases (Fator de impacto 2.167)

p16 PROTEIN EXPRESSION IN IDENTIFICATION OF ANAL INTRAEPITHELIAL LESIONS RELATED TO HUMAN PAPILLOMAVIRUS (HPV)

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ABSTRACT

Objective: The aim of the present study was to assess p16 protein expression in anal samples of HIV negative women with visible lesions by anoscopy. **Method:** A total of 65 patients, submitted to anoscopy, with lesions between the anal canal and the transition zone with the rectum were included in this study Cell collection and biopsies were performed. DNA was extracted to identify HPV by PCR using GP5+/6+ and MY09/11 primers. The biopsy fragments were subjected to routine hematoxylin-eosin staining for the histological assessment and immunohistochemistry reaction using anti-p16 antibody. **Results:** The histopathological patterns were grouped as follows: **I** - Negative for dysplasia: **Ia** - 52.31% (34/65) and **Ib** – benign changes of a proliferative nature 41.54% (27/65), **II** - Neoplasia 6.15% (4/65). Of the negative dysplasia cases, 93.44% (57/61) did not show a p16 marking. One Anal Intraepithelial Neoplasia (AIN) case (AIN I) as well as one AIN III case showed markings in one third and three thirds of the epithelium, respectively. The molecular analysis to identify HPV showed that 78.46% of the sample was positive. All lesions with a histological diagnosis of neoplasia were positive for HPV. **Conclusions:** The use of molecular biology and immunohistochemistry, associated with a histopathological exam, may provide a more accurate diagnosis and contribute to more effective AIN therapy.

Keywords: Anal Intraepithelial Neoplasia (AIN), Human papillomavirus (HPV), p16 protein, Polymerase Chain Reaction (PCR).

INTRODUCTION

A human papillomavirus (HPV) infection is the most common risk factor for the development of Anal Intraepithelial Neoplasia (AIN).[1] Other risk factors include a history of anal intercourse, HPV infections at other sites (vulva, vagina or cervix) and smoking. [2,3,4] Squamous cell carcinoma, the most common histological type of anal cancer, arises in areas of high grade AIN, as a consequence of chronic HPV infection, and appears to be related to a high load of virus in infected individuals.[5]

More than 150 HPV genotypes are known and approximately 40 are related to anogenital infections.[6] Currently, it is known that HPV can also be found in cancer of the penis, vagina, vulva, anus, perianal region, head and neck. HPV in the anogenital region can cause genital warts and lead to the development of lesions with a possible progression to cancer.[7,8]

The natural history of a HPV infection includes the following: transmission of the virus; the development of a persistent infection, and interaction with the immune system, which plays a crucial role in the progression from a lesion to cancer.[9] HPV infection tends to cause cancer in the area referred to as the "transformation zone". The anal region, as well as the cervix and tonsils are examples of areas prone to carcinogenesis by HPV. [10]

HPV has late structural genes (L1 and L2) that are involved in the structure of capsid and early functional genes (E1-E7) and related to viral replication processes.[11,12] The infection occurs in the basal layer of the squamous epithelium cell through a microtrauma. Malignant transformation in infected cells depends on the integration of the oncogenic HPV E6 and E7 genes in the host genome, the persistence of infection and low cellular immunity.[13] A negative feedback process resulting from this interaction activates the expression of p16 inhibitory proteins of the cyclin-dependent kinase complexes.[14,15]

p16 regulates the transition from the G1 phase to the S phase of the cell cycle [16] and is accumulated in the nuclei and cytoplasm of cells infected by HPV. Biomarkers for HPV infection, such as p16, have been shown to correlate with a histological grade of AIN. [17,18] The aim of the present study was to assess p16 protein expression in anal lesions of HIV negative women.

METHODS

Characterization and sample collection

The present study was conducted with 65 voluntary HIV negative female patients, aged between 18 and 60 years, who were submitted to anoscopy and attended the Cancer Hospital of Pernambuco. All patients had a history of lesions caused by HPV in the anogenital region. Cells were collected from the anal canal (from the transition zone to the rectum) with a gynecological brush and a biopsy was carried out.

HPV analysis

DNA was extracted from anal cells using the DNA IQ™ Casework Pro kit for Maxwell® 16, following the manufacturer's instructions. To ensure the quality of DNA extraction, the reporter gene β-globin for PCR, amplifying a region of 268bp using specific primers (5'-CAACTTCATCCACGTTCACC-3'/5'-GAAGAGCCAAGGACAGGTAC-3') was used. Ultrapure water was used as a negative control and DNA extracted from human blood was used as a positive control. The amplification was prepared with a final volume of 12.5μL containing: 1μL DNA, 1uL of each primer (10pmoles), 3.25μL ultrapure water and 6.25μL GoTaq Green of Master Mix (Promega®). The PCR conditions were as follows:

initial denaturation for 10 minutes at 94°C followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute. The final extension step was performed at 72°C for 7 minutes.

The presence of HPV in biological samples was analyzed by PCR using consensus primers for the region of the HPV L1 gene: GP5+/6+ (5'-TTTGTTACTGTGGTAGATACTAC-3'/5'-GAAAAATAAACTGTAAATCATATTC-3') which amplifies a fragment of 150bp; and MY09/11 (5'-CGTCCMARRGGAWACTGAT-3'/5'-GCMCAGGGWCATAAYAATGG-3') which amplifies a fragment of 450bp from the same gene. The final volume of the reaction was 12.5µL containing: 1µL of extracted DNA, 1µL of each primer (10pmoles), 3.25µL ultrapure water and 6.25µL GoTaq Green of Master Mix (Promega®). The positive control used was pBR322.HPV16 plasmid whereas the negative control used was ultrapure water. Amplification conditions were as follows: (i) 94°C for 3 minutes, (ii) 34 cycles of denaturation at 95°C for 1 minute, annealing for 1 minute and extension at 72°C for 1 minute, (iii) final extension at 72°C for 10 minutes. MY09/11 primers annealed at 55°C, whereas GP5+/6+ annealed at 45°C. The amplifications products were revealed by 1% agarose gel electrophoresis with ethidium bromide.

Histology and immunohistochemistry

Tissue fragments from the biopsies were fixed in 10% buffered formalin and embedded in paraffin. Histological slices were obtained (4µm thickness) and stained with hematoxylin-eosin (HE) or used in the immunohistochemical test.

Immunohistochemical staining of tissue sections was done using the CINtec® Histology kit to detect tissue of $p16^{INK4a}$ (Biogen). The histological sections were mounted on silanized slides, deparaffinized and incubated in antigen retrieval solution (1:10) previously

heated in a steamer at 95º C for 10 minutes. After cooling to room temperature (RT), the slides were washed with wash buffer (1:10), dried and 50µl of reagent was added to block endogenous peroxidase in tissues and then incubated for 5 minutes at RT. After rinsing in wash buffer, tissue sections were incubated for 30 minutes at RT with 50µL of the primary monoclonal antibody anti-p16 (clone E6H4). Tissue sections were washed twice in wash buffer and incubated for 30 minutes at RT with visualization reagent (polymer reagent conjugated with horseradish peroxidase). After washing in wash buffer, peroxidase activity was detected by incubating tissue sections for 10 minutes at RT with DAB Chromogen (3.3'-diaminobenzidine chromogen solution). Tissue sections were counterstained with Harris´s hematoxylin.

Histological and immunohistochemical assessment

The histopathological patterns were grouped and classified as follows: *I - Negative for dysplasia:* **Ia** - *without significant changes (Normal), benign reactive or inflammatory changes* and **Ib** – *benign changes of proliferative nature* (including condyloma, polyps and hyperplasia) and **II** - Neoplasia *(Anal Intraepithelial Neoplasia grade I (low-grade) II and III (high-grade).*

The p16 positivity was defined by the presence of nuclear and/or cytoplasmatic staining. The classification criteria were based on the thickness of the anal epithelium marked: one third of inferior epithelium (basal layer), between one third and two thirds of the epithelium, or three thirds, corresponding to the entire thickness of the epithelium. The pattern of staining was considered as focal or continuous.

RESULTS

Profile of patients

In the present study, there was a high prevalence of patients aged between 26 to 35 years (36.92% - 24/65). In relation to sexual activity, 36.92% of the women had more than five sexual partners (24/65) and 64.41% (42/65) reported a history of anal intercourse. A total of 98.46% (41/42) did not use condoms during anal intercourse.

Histology and Immunohistochemistry

The results obtained were as follows: *I - Negative for dysplasia:* ***Ia*** *- without significant changes (Normal), benign reactive or inflammatory changes* - 52.31% (34/65); ***Ib*** *– benign changes of a proliferative nature* (including condyloma, polyps and hyperplasia) – *41.54% (27/65)* and ***II*** *- Neoplasia (Anal Intraepithelial Neoplasia grade I (low-grade) I/II and III (high-grade)* - 6.15% (4/65).

In 93.44% (57/61) of the negative dysplasia cases, and one case of AIN I, there was an absence of p16 staining (Figure 1a). However, the other four cases showed markings for p16, restricted to the basal layer of epithelium and classified histologically as Ib (inflammation) markings a with three thirds focal pattern.

Nuclear and/or cytoplasmatic markings were observed in 75% (3/4) of neoplasia cases with staining restricted to the basal layer (AIN I) or marking the entire thickness of the anal epithelium (AIN III) (Figure 1c). One case of AIN I/II exhibited different glandular markings to the criteria used for other cases (Table 1).

Molecular Analysis

According to the molecular analysis to identify HPV, 51 of the 65 samples analyzed by PCR (78.46%) exhibited a positive result. Only 72.30% (47/65) of the samples were considered to be of sufficient quality for HPV analysis by PCR, observed by the β-globin primer. Using the GP5+/6+ primer to identify HPV, a positivity rate of 68.08% (32/47) was obtained whereas the MY09/11 primer found a positivity rate of 36.17% (17/47). Twenty-one samples exhibited nonspecific bands and were thus considered indeterminate to detect HPV-DNA.

A comparison was made between the results of the histological exam and the identification of HPV by PCR. All lesions with a histological diagnosis of neoplasia, independent of the degree, were positive for HPV. In patients diagnosed with lesions of a proliferative nature, 85.18% (23/27) also exhibited positivity for HPV (Table 2).

DISCUSSION

The HPV shows tropism for the skin and mucous membranes[2]. The anal canal, as well as the entire anogenital tract, can be a part of the HPV circuit or may even be a reservoir for the virus due to the presence of squamous epithelium.[4]

HPV infection is a risk factor for the development of squamous premalignant lesions and malignant lesions in the anal area. The interaction of viral oncoproteins, E6 and E7, with cell cycle regulatory proteins, p53 and pRb, and the relationship between pRb and the transcription factor E2F, can lead to the activation of cyclin-dependent kinases (CDK 4 and CDK6) which, in turn, lead to the expression of associated protein kinases, such as p16.[19,20] p16 expression is up-regulated in the transformation caused by infection with oncogenic

HPV, and showed itself to be a sensitive marker for squamous intraepithelial lesions of the female genital tract.[21,22]

The majority of studies focus on marking with p16 in cervical samples.[23,24] In cases of grade II and III Cervical Intraepithelial Neoplasia (CIN), a greater p16 expression occurs. Positivity in cases of CIN I, although uncommon, appears to have a risk of progression to malignancy.[24,25]

According to Lu et al.,[26] there is a similarity in markup with p16 in both anal and cervical samples. In their study using anal samples, Lu et al. observed strong and diffuse nuclear staining (with some cytoplasmatic staining) in all cases of anorectal squamous cell carcinoma.

In a study performed by Kreuter et al.,[27] it was demonstrated that p16 protein identification through immunohistochemistry is an excellent additional tool in the diagnosis of high grade anal dysplasia. In normal anal mucosa, the protein expression is only null or positive in a number of isolated cells. The findings of the present study are in agreement with those of Kreuter et al.,[27] in relation to the fact that most cases of negative histological diagnosis showed an absence of p16 marking, and when present, it was restricted to the basal layer, which may suggest that the lesion has a higher potential progression. Furthermore, Pirog et al.[28] stated that p16 is a highly sensitive and specific marker for high grade AIN, in relation to low-grade lesions, and thus can aid in the classification of neoplasia related to HPV.

The prognosis of anal squamous cell carcinoma is substantially affected by the disease stage. The early detection and treatment of precursor lesions entails benefits to the patient.[27] Bean et al.[17] revealed how routine p16 marking can assist in confirming a histological diagnosis or in detecting intraepithelial lesions that appear as negative using the histological

method. This demonstrates the importance of combining the histological method with other methods to assist in the confirmation of results.

The diagnosis and grading of AIN is straightforward in some anal biopsies. However, not infrequently, overlapping morphologic features and/or inflammation with associated epithelial atypia can make it difficult to distinguish non-HPV related squamous hyperplasia and other reactive conditions.[18]

Studies comparing p16 marking with the degree of anal lesions were predominantly composed of HIV-positive patients.[27,29] Although anal cancer is a rare disease in the general population, it was demonstrated that the incidence of anal cancer is higher in certain high risk groups, such as MSM (Men who have sex with men) and immune suppressed individuals, including those with HIV.[30]

Kreuter et al.[27] conducted a study with a HIV-positive population and highlighted male homosexuals as a risk group. Both studies found a higher frequency of anal lesions of a higher degree, such as AIN. In the present study, more severe lesions were not prevalent, although the type of population studied, composed of HIV-negative women, must be considered.

With regard to the molecular analysis of HPV, the index of positive samples for the virus was high, considering that most samples presented positivity for one of the primers used. This is due to the fact that the study population had a history of HPV infection at another site, and that the presence of the virus in the anal site does not necessarily indicate the presence of a clinical lesion with higher severity. It is important to analyze the viral type involved in this infection.

Most patients who reported having anal intercourse did not use condoms, which increases the probability of HPV infection and reveals a lack of knowledge about the risk of infection in the anal region among the population of the present study. This shows the need

for dissemination about infections in the anal region, similar to that which occurs related to the cervical region, as well as the implementation of periodic screening sessions in healthcare systems.

The histological exam is considered a gold standard for the diagnosis of AIN. However, considering the results of the present study, it can be stated that the utilization of additional tools, such as immunohistochemistry and PCR, is important to optimize the diagnostic accuracy for AIN.

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Figure 1

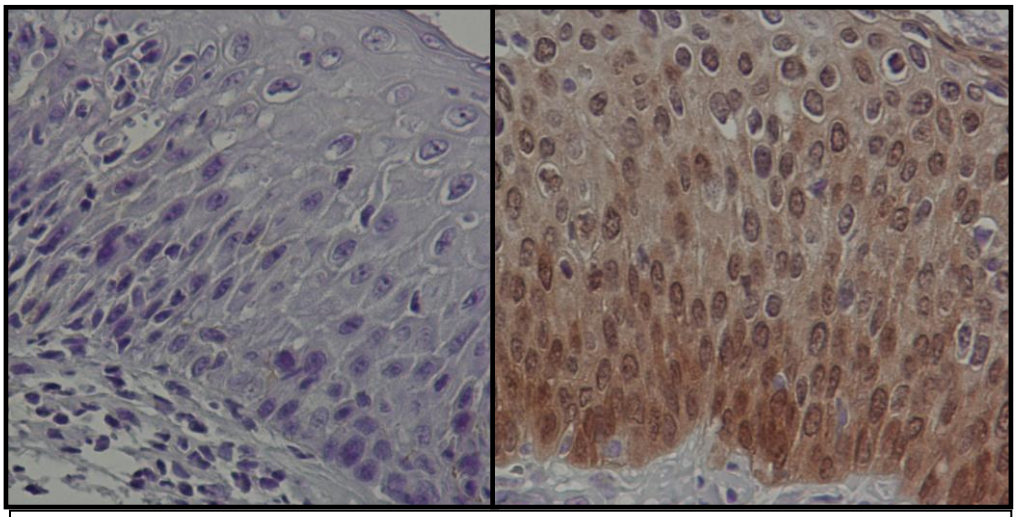

Figure 1 - **A**: Anal epithelium negative for dysplasia (*without significant changes*) with absence of p16 markings; B: p16 markings in all thickness of the anal epithelium (AINIII). (40x)

Table 1 - p16 protein expression in identification of the degree of anal intraepithelial lesions

Histological diagnosis (n)	**p16 Expression**				
	Negative	**1/3**	**2/3**	**3/3**	**Indeterminate**
I: Negative					
Ia: Normal, benign reactive or inflammatory changes (n = 34)	**28**	**02**	**-**	**01°**	**03**
Ib: Proliferative changes (n = 27)	**23**	**01**	**-**	**-**	**03**
II: Neoplasia					
AIN I (n = 02)	**01**	**01**	**-**	**-**	**-**
AIN I/II (n = 01)	**-**	**-**	**-**	**01***	**-**
AIN III (n = 01)	**-**	**-**	**-**	**01**	**-**
TOTAL	**52**	**04**	**-**	**03**	**06**

° Focal pattern
*Glandular pattern

Table 2 - Comparison between PCR and histological diagnosis results. Positive results for PCR were considered when at least one set of primers was positive

Histological diagnosis (n)	**PCR**	
	Positive	**Negative**
I: Negative		
Ia: Normal, benign reactive or inflammatory changes (n = 34)	24	10
Ib: Proliferative changes (n = 27)	23	04
II: Neoplasia (n = 04)	04	-

4.2 Artigo Original 2

Revista Journal of Clinical Virology (Fator de impacto: 3.607)

IDENTIFICATION AND GENOTYPING OF HUMAN PAPILLOMAVIRUS IN ANAL SAMPLES OF WOMEN HIV-NEGATIVES

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ABSTRACT

Objective: Analyze molecular methodologies for HPV-DNA detection in anal cell from patients with anal lesions, using Polymerase Chain Reaction (PCR) and PapilloCheck®. **Methods:** The study was conducted with 82 patients with cervical lesions, attended in service of Pelvis of Cancer Hospital of Pernambuco, submitted to anoscopy magnification to observe anal lesions. Cell samples were collected from anal canal and submitted to DNA extraction. HPV detection was performed by PCR, using the primers GP5+/6+ and MY09/11. HPV genotyping was identified by PapilloCheck® HPV-screen DNA-chip. **Results:** HPV-DNA was detected in 76.19% (48/63) by PCR technique. GP5+/6+ primers showed the highest positivity rate 66.67% (42/63), while MY09/11 primers detected HPV-DNA in only 46.03% (29/63) with other 21 samples showing nonspecific amplification. PapilloCheck® showed 50% positivity with identification of 20 HPV genotypes, being HPV6 (18.84%) the most prevalent, followed by HPV16 (15.94%) and HPV53 (10.14%). Multiple infections were observed in 24.64%, showing variation between 2-8 virus types. 34.15% samples were considered as negative for HPV, despite 39.28% (11/28) were positive in PCR, indicating that some HPV genotypes not included in Papillocheck® setting could be infecting anal samples. 15.85% (13/82) samples presented sampling error during genotyping process, revealing that the sample quality interferes directly in the results due to factors such as amount of DNA and/or contaminants in the sample. **Conclusion:** PapilloCheck® was able to identify multiple HPV in anal cells. However, PCR technique was more effective in HPV identification being cheaper for routine analysis in anal cells from women with cervical lesions.

Key-words: Anal Intraepithelial Neoplasia (AIN), human papillomavirus, genotyping, Polymerase Chain Reaction (PCR).

INTRODUCTION

Anal cancer is a rare malignancy that accounts for 2% of all colorectal malignancy. In the United States 5,820 new cases and 770 deaths from anal cancer were estimated in 2011[1]. In Brazil, approximately 1,617 new cases of anal cancer were diagnosed in 2009[2].

Squamous cell carcinoma is the most common histological type of anal cancer, being consequence of chronic infection by HPV and seems to be related to the high load of virus infection[3]. HPV infection is the most common risk factor for the development of Anal Intraepithelial Neoplasia (AIN)[4]. Other risk factors include history of anal intercourse, history of HPV at other sites (vulva, vaginal or cervical) and smoking[5,6,7]. A significant association has also been found with carcinoma of the anus, which HPV can be detected in 84% of the cases. Focused just in women, the prevalence of HPV in the anal cancer is even higher than 90.8% [8].

Currently, it is known that HPV can be found in cancer of the penis, vagina, vulva, head, anus and perianal region[9]. The anal canal, as well as all genital tract, can be part of the HPV circuit or even be a reservoir for the virus, due to the presence of squamous epithelium[7]. It should be taken into consideration the possibility of self-contamination by injuries at other sites induced by HPV[7,8].

Because anogenital HPV infection is a possible precursor of cancer, there is a concern in carrying out their diagnosis through screening, with the aim of prevent malignant transformation, as performed in gynecology[10,11]. The aim of this study was to analyze the efficiency of molecular methodologies for HPV-DNA detection in anal samples of the patients with anal lesions, using Polymerase Chain Reaction (PCR) and PapilloCheck®.

METHODOLOGY

Characterization and sample collection

The survey was conducted with 82 voluntary female patients, aged between 18 and 60 years, attended the service of the Pelvis Cancer Hospital of Pernambuco – Brazil. All patients were HIV negative, presented cervical lesion and were submitted to anoscopy magnification for anal injuries diagnosis. All patients had a history of HPV infection in the anal region or other site (cervical, vulvar and perianal). Cell samples were collected in the region of anal canal from the transition zone to the rectum using gynecological brush. Samples were maintained in PBS buffer under 4^{o}C up to the analysis.

Extraction of DNA samples

DNA was extracted from anal cells using DNA IQ™ Casework Pro kit for Maxwell® 16, following manufacturer's instructions. To ensure the quality of DNA extraction it was used the reporter gene β-globin for PCR, amplifying a region of 268bp using specific primers (5'-CAACTTCATCCACGTTCACC-3'/5'-GAAGAGCCAAGGACAGGTAC-3'). Ultrapure water was used as negative control and DNA extracted from human blood was used as positive control. The amplification was prepared for final volume of 12.5µL containing: 1µL DNA, 1uL of each primer (10pmoles), 3.25µL ultrapure water and 6.25µL GoTaqGreen of Master Mix (Promega®). The PCR conditions were: initial denaturation for 10 min at 94°C followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min. The final extension step was performed at 72°C for 7 min. The amplifications products were revealed by 1% agarose gel electrophoresis with ethidium bromide.

HPV identification by PCR

The presence of HPV in biological samples was analyzed by PCR using consensus primers for the region of the HPV L1 gene, GP5+/6+ (5'-TTTGTTACTGTGGTAGATACTAC-3'/5'-GAAAAATAAACTGTAAATCATATTC-3') that amplify a fragment of 150bp and MY09/11 (5'-CGTCCMARRGGAWACTGAT-3'/5'-GCMCAGGGWCATAAYAATGG-3') that amplify a fragment of 450bp from the same gene. The final volume of the reaction was 12.5μL containing: 1μL DNA extracted, 1μL of each primer (10pmoles), 3.25μL ultrapure water and 6.25μL GoTaqGreen of Master Mix (Promega®). The positive control used was pBR322.HPV16 plasmid and negative control, ultrapure water. Amplification conditions were performed as follows: (i) 94°C for 3 minutes, (ii) 34 cycles of denaturation at 95°C for 1 minute, annealing for 1 min and extension at 72°C for 1 minute, (iii) final extension at 72°C for 10 min. MY09/11 primers annealed at 55°C, whereas GP5+/6+ annealed at 45°C. The amplifications products were revealed by 1% agarose gel electrophoresis with ethidium bromide.

HPV genotyping

HPV genotyping was performed using PapilloCheck® HPV-screen DNA-chip (Greiner Bio-one®), capable to identify 24 HPV types, being 06 low risk genotypes (06, 11, 40, 42, 43, 44/55) and 18 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82). The PapilloCheck® assay is based on the PCR amplification of E1 gene from HPV resulting 350 nucleotides fragment.

RESULTS

A total of 82 anal samples were analyzed by DNA extraction and the quality of DNA was observed by β-globin PCR. However, only 76.82% (63/82) of samples were considered with good quality for HPV analysis by PCR. Evaluating the HPV detection by PCR technique with both primers set, it was obtained a positivity rate of 76.19% (48/63) and GP5+/6+ primers showed the highest positivity rate 66.67% (42/63). MY09/11 primers were able to detect HPV-DNA in 46.03% (29/63), but another 21 samples showed nonspecific bands, being considered undetermined for HPV confirmation (Table 1). Despite 19 samples were negative for β-globin amplification, 13 samples were positive for HPV.

Among all 82 anal samples analyzed by PapilloCheck®, 28 were negative for HPV. In 41 HPV-positive samples (50%), 20 genotypes were identified, being 05 low-risk HPV (06, 11, 42, 43 e 44/55) and 15 high-risk HPV (16, 31, 33, 35, 39, 45, 51, 53, 56, 58, 59, 66, 68, 70, 82). A higher prevalence of HPV6 (18.84%), HPV16 (15.94%) and HPV53 (10.14%) was observed in this study. Multiple infections were observed in 17 samples (24.64%), showing variation between 2-8 virus types (Table 2). HPV16 was present in 52.94% (9/17) of multiple infected samples, followed by HPV6 (35.94%), HPV53 (29.41%) and HPV68 (29.41%).

A total of 13 samples were unable to be analyzed in PapilloCheck® due to the quality of the sample, despite 05 samples were positive for β-globin amplification and 11 were determined as positive for HPV by GP5+/6+, while 07 were positive for MY09/11 (Table 3). Otherwise, among 19 negative samples for β-globin, 08 were unable to amplify in the HPV-screen chip.

DISCUSSION

Females with cervical HPV infection have a three-fold increased risk of concurrent anal infection[12,13]. Despite recommendations of AIN screening in high-risk populations, the optimal screening methods have not been established. High-resolution anoscopy (HRA) is an excellent and easily performed means of detecting squamous intraepithelial lesion high grade (HSIL), but resources for HRA are limited[14].

Anal cytology and molecular tests have been analyzed as possibilities for better identifying HPV in patients[14]. PCR method is more sensitive to identify the virus in reduced samples[15,16]. Regarding the type of anal sample used, fresh cells tend to result in higher HPV positivity and broader spectrum of detected HPV types compared to tissue biopsies, once collected cells represent the whole anal canal[17].

The sample type and quality of DNA extraction may influence the PCR reaction. In the harvest of anal cells, compared with cervical cells, the number of cells is reduced by the presence of a thicker epithelium. Thus the amount of DNA obtained in some samples may not be sufficient for a good quality of the reactions. These facts are related to the 19 samples considered unsatisfactory for PCR analysis in our study, as well as 13 “sample failed” result in PapilloCheck®. Therefore, concordance in HPV presence by both methods was considered low, once occurred in only 39/82 (47%) samples.

PCR amplification is considered the most sensitive method for the detection of HPV DNA[18]. The most commonly used consensus primers designed for broad spectrum coverage of HPV types are: (i) GP5+/6+, able to identify up to 37 HPV genotypes and (ii) MY09/11 with identification spectrum of up to 45 HPV types[19,20]. However, these primers may vary in PCR efficiency and amplicons production due to intrinsic differences in primer sequence and other factors, i.e. DNA template quality/quantity[21,22].

In this study the amplification using the primer GP5+/6+ showed high efficiency for HPV-DNA identification in anal samples. MY09/11 primer should not be considered for this purpose due to nonspecific amplifications in some samples, which could be related to other microbial infections in anal canal.

Didelot (2011)[23] evaluating 59 female anal scrapes through PapilloCheck® found 24 HPV genotypes in 74.6% of samples, being higher than data found in our study (59.42%), with HPV16, HPV39 and HPV53 appearing as most frequent. A study conducted with 289 male anal samples using Hybrid Capture 2 HPV test found HPV16 (21.69%), HPV6 (18.16%), HPV11 (16.27%)[24]. Another study, conducted in Argentina with 119 male anal samples, found prevalence of HPV16 (30.1%), HPV42 (18.4%) e HPV81 (17.5%)[25]. Curiously, HPV6 was the most frequent genotype in single infection in our study not in accordance with the literature that found HPV16 in most samples, which seems to be related to the geographical parameters as observed for HPV in cervical samples[26].

The frequency of multiple infection in anal cells in our study (24.64%) was higher than Didelot (2011)[23] findings (18.4%). HPV16 was the most frequent in multiple infections (9/17) and together with other two high-risk, HPV53 (5/17) and HPV68 (5/17), are involved most often in multiple infection with more than three viral types. The cases of multiple infection involving the HPV6 have a lower variability of viral types, however HPV6 is associated with one or more high-risk genotype in 83.34% cases. The risk of progression of HPV-associated lesions to precancerous or cancerous lesions varies according to the HPV types[27,28]. Orlando et al, (2009)[24] showed that mixed infections from multiple HPV genotypes are more likely due to high-risk HPV. In fact, all samples with multiples infection showed al least one high-risk type, which could contributes to the development of HPV infection, increasing the risk of progression to neoplasia. In this way, the implementation of a screening program for HPV infections in anal region from women with cervical lesions could be an

important approach with the aim of prevents anal cancer and persistent infections that leads to cervical cancer.

Further studies should be performed using PapilloCheck®, mainly due to the lacking of information about viral prevalence anal region according geographical disposal. However, this HPV-screen DNA-chip probably does not cover some important HPV types that infect this tissue, once PCR test was more efficient for HPV detecting in anal samples. Therefore, PCR tests could be a good alternative for public health screening program, preventing the evolution of HPV infection to anogenital cancers.

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Table 1. Agreement between GP5+/6+ results and MY09/11 for HPV detection in anal cell samples from women with cervical lesions.

	GP5+/6+	
	Positive	**Negative**
MY09/11		
Positive	27	07
Negative	11	05
Indeterminate	16	06

Table 2. Distribution of HPV genotypes in 69 anal scrapes as detected by the PapilloCheck® assay.

HPV genotypes	Frequency (%)	Multiple infection (n)	Single infection (n)
06*	18.84	06	07
11*	2.89	02	-
16	15.94	09	02
18	-	-	-
31	1.44	01	-
33	1.44	-	01
35	1.44	01	-
39	4.34	03	-
40*	-	-	-
42*	2.89	01	01
43*	1.44	01	-
44/55*	2.89	01	01
51	4.34	02	01
52	-	-	-
53	10.14	05	02
56	5.79	03	01
58	5.79	02	02
59	8.69	04	02
66	5.79	01	03
68	7.24	05	-
70	5.79	02	01
73	-	-	-
82	2.89	02	-
Negative	40.58	-	-

***** indicate the 06 low-risk HPV detected by the system

Table 3. Agreement between PCR and PapilloCheck® results for HPV detection in anal cell samples from women with cervical lesions. (Positive results for PCR were considered when at least one primers set was positive).

	PCR (MY/GP)	
	Positive	**Negative**
PapilloCheck®		
Positive	39	02
Negative	11	17
Indeterminate	11	02

CONSIDERAÇÕES FINAIS

O diagnóstico histológico é considerado como padrão-ouro para o diagnóstico de NIA, no entanto, é fundamental a inserção de métodos adicionais, como imunohistoquímico e molecular, com o intuito de otimizar a precisão dos resultados e prevenir a evolução da infecção para o câncer.

O método imunohistoquímico utilizando a proteína p16 mostra-se mais eficiente em lesões de alto grau. Porém, são necessários estudos com uma maior quantidade de casos de neoplasias para uma melhor análise dos diferentes níveis de expressão da p16 relacionados ao grau da lesão.

A utilização de primers específicos na identificação do HPV em amostras anais mostrou-se eficaz quando utilizado o primer GP5+/6+, podendo assim ser empregado como método auxiliar na avaliação de lesões intraepiteliais anais. O primer MY09/11 não foi eficiente para identificação neste tipo de amostra.

O método de genotipagem através do PapilloCheck® mostrou-se eficiente para identificação do HPV em células anais, porém devido ao alto custo, torna-se inviável sua utilização em rotinas de rastreamento de pacientes que fazem parte de grupos de risco.

São necessárias pesquisas futuras que envolvam um maior número de pacientes para uma melhor visão da prevalência dos genótipos do HPV na região anal, principalmente os potencialmente oncogênicos, de acordo com a disposição geográfica.

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APÊNDICES

Apêndice A

Tabela. Perfil das pacientes atendidas no Hospital de Câncer de Pernambuco submetidas ao exame de anuscopia de magnificação.

PARÂMETRO	NÚMERO DE PACIENTES	PERCENTUAL
	Faixa Etária	
18 – 25 anos	**09**	**13,85%**
26 – 35 anos	**24**	**36,92%**
36 – 45 anos	**15**	**23,08%**
46 – 60 anos	**17**	**26,15%**
Total	**65**	**100%**
	Parceiros Sexuais	
Um	**14**	**21,54%**
Dois	**09**	**13,85%**
Três	**14**	**21,54%**
Quatro	**04**	**6,15%**
Cinco ou mais	**24**	**36,92%**
Total	**65**	**100%**
	Intercurso anal	
Sim	**42**	**64,61%**
Não	**23**	**35,39%**
Total	**65**	**100%**
	Preservativo no intercurso anal	
Sim	**1**	**1,54%**
Não	**41**	**98,46%**
Total	**42**	**100%**

Apêndice B

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

O presente termo refere-se a um convite a Sra. _____ a participar da pesquisa intitulada: "Imunoexpressão da proteína p16 em lesões intra-epiteliais anais relacionadas com o Papillomavirus humano (HPV)". A pesquisa será coordenada pela bióloga Maria Julliana Galvão Nunes e pela médica pesquisadora Maria das Graças de Fátima Cavalcante Castor, contatadas através do telefone: (81) 96728083; e-mail julliana_gnunes@yahoo.com.br ou gracafccastor@hotmail.com ou endereço profissional: Laboratório de Imunopatologia Keizo Asami situado na Avenida. Prof. Moraes Rego, S/N, Cidade Universitária, Recife/PE CEP: 50670-901, telefone: (81)2126-8485.

A pesquisa será realizada através do exame de colposcopia anal, que tem o objetivo de observar o canal anal através da introdução do anuscópio, instrumento rígido em forma de especulo (semelhante a forma de um tubo), lubrificado, que permite a avaliação do canal anal através da visualização, tendo a finalidade de verificar a existência de lesões pré-cancerosas ou câncer e na presença de "manchas" será realizada a retirada de um pequeno fragmento sob anestesia; assim como realizar a coleta de sangue para o teste de HIV (vírus da AIDS).

Com relação aos riscos, a realização dos exames apenas provoca desconforto, quando da introdução dos instrumentos ou mesmo a picada da agulha da anestesia ou coleta de sangue. Os benefícios pela participação na pesquisa é que os procedimentos realizados são necessários tendo em vista a possibilidade de cura, caso seja detectado alguma lesão pré-cancerosa. No caso da existência de lesão pré-cancerosa ou câncer, a paciente será tratada pelos médicos do Serviço de Pélvis do Hospital de Câncer do Recife.

Não haverá nenhum incentivo financeiro pela participação na pesquisa, sendo esta com finalidade exclusiva de colaborar para o sucesso da pesquisa com objetivos estritamente acadêmicos. A participação na pesquisa poderá ser interrompida, se assim desejar, sem sofrer nenhum constrangimento ou qualquer prejuízo para o seu tratamento, seja tratamento clínico ou cirúrgico.

As informações oferecidas pela participante serão submetidas às normas de ética em pesquisa (CONEP) do Conselho Nacional de Saúde, do Ministério da Saúde. Em caso de dúvida poderá contatar ainda o Comitê de Ética em Pesquisa da Universidade Federal de

Pernambuco, situado na Av. Prof. Moraes Rêgo, s/n - 1º andar, Cidade Universitária CEP: 50670-901 Recife/PE, Brasil. Telefone/Fax do CEP: (81) 2126-8588. A identidade do participante será mantida em sigilo e os resultados da pesquisa poderão ser apresentados em eventos e publicações científicas.

A pesquisadora disponibilizará uma cópia assinada deste Termo de Consentimento Livre e Esclarecido, conforme recomendações da Comissão Nacional de Ética em Pesquisa (CONEP).

Assim se a Sra. aceitar o convite para participar da pesquisa, por favor, preencha os espaços abaixo:

Eu,_____, RG_____, fui devidamente esclarecida do projeto de Pesquisa acima citado e aceito o convite para participar.

Recife,_____de _____20___.

1_____

2_____

Testemunhas

Assinatura da pesquisadora_____

Assinatura do pesquisado _____

Apêndice C

QUESTIONÁRIO

DATA:____/____/_____.

NOME:_____ N°.:_____

IDADE: _____

REGISTRO: _____

HIV:_____ DATA: _____/_____/_____.

PARCEIROS N°.:_____

INTERCURSO ANAL ( )SIM ( )NÃO

CAMISINHA NO INTERCURSO ANAL ( )SIM ( )NÃO

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RESULTADO DO EXAME HISTOPATOLÓGICO:

ANEXOS

Anexo A

Parecer do Comitê de Ética em Pesquisa

SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE PERNAMBUCO
Comitê de Ética em Pesquisa

Of. Nº. 360/2010 - CEP/CCS

Recife, 21 de dezembro de 2010

Registro do SISNEP FR – 358589
CAAE – 0029.0.447.172-10
Registro CEP/CCS/UFPE Nº 280/10
Titulo: **Imunoexpressão da proteína p16ink4a em lesões intra-epiteliais relacionadas com o papilomavirus humano(HPV).**
Pesquisador Responsável: Maria Juliana Galvão Nunes

Senhor(a) Pesquisador(a):

Informamos que o Comitê de Ética em Pesquisa Envolvendo Seres Humanos do Centro de Ciências da Saúde da Universidade Federal de Pernambuco (CEP/CCS/UFPE) registrou e analisou de acordo com a Resolução N.º 196/96 do Conselho Nacional de Saúde, o protocolo de pesquisa em epígrafe, liberando-o para início da coleta de dados em 21 de dezembro 2010.

Ressaltamos que a aprovação definitiva do projeto será dada após a entrega do relatório final, conforme as seguintes orientações:

a) Projetos com, no máximo, 06 (seis) meses para conclusão: o pesquisador deverá enviar apenas um relatório final;
b) Projetos com períodos maiores de 06 (seis) meses: o pesquisador deverá enviar relatórios semestrais.

Dessa forma, o ofício de aprovação somente será entregue após a análise do relatório final.

Atenciosamente

Prof. Geraldo Bosco Lindoso Couto
Coordenador do CEP/CCS / UFPE

A
Mestranda Maria Juliana Galvão Nunes
Programa de Pós-Graduação em Patologia CCS/UFPE

Anexo B – Instruções para publicação na Revista Paraense de Medicina

ISSN 0101-5907
versão impressa

INSTRUÇÕES AOS AUTORES

- Orientações gerais
- Formatação de artigos
- Endereço para correspondência

Orientações gerais

A **Revista Paraense de Medicina** aceita para publicação, trabalhos científico-culturais da área de saúde, sob forma de: **Artigo original**; **Atualização/Revisão**; **Relato de caso**; **Artigos especiais e sobre a linguagem médica**; **Nota prévia** e **Carta ao editor**.

Os artigos devem ser envidados em CD-RW Rewritable 1X-12X 700MB ou disquete 3 ½ polegadas, com dois textos originais, impressos em papel A4, digitados no Windows 98 e Microsoft Word versão 2000 XP, com espaço simples, fonte TNR-12 e duas colunas. O SUMMARY, fonte 11 e referências fonte 10, em uma coluna.

As tabelas e quadros, incluidas no texto, devem possuir legenda na parte superior, fonte TNR 10, identificados com números romanos, indicando o que, onde e quando do tema, com nota de rodapé TNR 9. Os gráficos, fotos, esquemas, etc. são considerados como figuras, recebendo identificação inferior, TNR 10, seqüêncial único em algarismos arábicos.

Fotografias deverão ser enviadas em tamanho 9x13cm, preto e branco com boa qualidade e com as estruturas a serem identificadas. As figuras de anatomia, histopatologia e endoscopia poderão ser coloridas.

Os autores são responsáveis pelos conceitos emitidos e devem atentar à seriedade e qualidade dos trabalhos, cujos dados devem receber tratamento estatístico, sempre que indicados.

Encaminhar, aos editores da RPM, os artigos com carta modelo, com timbre da Instituição e assinada pelos autores para devida avaliação pelo Conselho Editorial.

Todo trabalho com investigação humana e pesquisa animal deve ser acompanhado da aprovação prévia da Comissão de Ética em Pesquisa da

instituição, onde se realizou o trabalho, conforme recomenda a Declaração de Helsinki (de 1975 e revisada em 1983) e as Normas Internacionais de Proteção aos Animais e a Resolução n° 196/96, do Ministério da Saúde, sobre pesquisa em seres humanos.

Os artigos enviados à RPM não podem ser publicados em outras revistas biomédicas.

Formatação dos artigos

Editorial

É o artigo inicial de um periódico. Comenta assunto atual de interesse à área de saúde, editoração, metodologia científica ou temas afins.

Artigo original

Aborda temas de pesquisa observacional ou experimental, transversal (incidência ou prevalência), horizontal ou longitudinal (retrospectiva ou prospectiva), estudo randomizado ou duplo cego, máximo de 6 a 10 laudas. A pesquisa bibliográfica acompanha todo trabalho bio-médico.

1) Título e subtítulo (se houver), em português, TNR fonte 12, com tradução para o inglês, fonte 11, centralizados.

2) Nome completo dos autores, máximo de 6, com sobrenome em letras maiúsculas, TNR 11, também, centralizados.

3) No rodapé da 1ª página, citar a instituição onde foi realizado o trabalho e titulação dos autores, TNR 10, numerada conforme a seqüência dos autores.

4) O resumo deve ser escrito em parágrafo único, itálico, TNR 12, contendo: objetivo, método (casuística e procedimento), resultados (somente os significantes) e conclusão ou considerações finais.

5) Descritores: citar no máximo 5 e em ordem de importância para o trabalho, conforme relação do Index Medicus.

6) Introdução: mostra a hipótese formulada, atualiza o leitor na relevância do tema sem divagação e termina com o objetivo do trabalho.

7) Método: descreve a casuística, amostra ou material e procedimentos utilizados para o trabalho.

8) Resultados: constituído por, no máximo, 6 tabelas numeradas, com legenda superior (TNR 10) e fonte de informação abaixo (TNR 9), acompanhadas ou não de gráficos. Não fazer comentários, reservando-os para o ítem Discussão.

9) Discussão: compara os resultados da pesquisa com os da literatura referenciada, de

maneira clara e sucinta.

10) Conclusões ou considerações finais sobre os resultados da pesquisa ou estudo, de forma concisa e coerente com o tema.

11) Summary: versão do resumo do trabalho para a língua inglesa, TNR 11, itálico. Deve constar o título, nomes dos autores e os itens superpostos.

12) Key words: segundo o DECS e na língua inglesa.

13) Referências: devem ser atualizadas, (TNR 10), obedecendo o estilo Vancouver, em ordem numérica conforme a citação no texto, máximo de 30 citações.

Exemplificando

Artigos:
TEIXEIRA, JRM .- Efeitos analgésicos da *Maytenus guianensis*: estudo esperimental, *Rev. Par. Med*. 2001, 15(1): 17-21
O nome do periódico é de forma itálica.

Livro e monografia:
COUSER, WG – Distúrbios glomerulares. In:CECIL – *Tratado de Medicina Interna*, 19 ed. Rio de Janeiro: Ed. Guanabara, p. 477-560, 1993

Internet:
MOKADDEM, A (e colaboradores). Pacemaker infections, 2002. Disponível em http:/www.pubmed.com.br – Acessado em ....

As qualidades básicas da redação científica são: concisão, coerência, objetividade, linguagem correta e clareza.

Atualização/revisão

Obedece o mesmo padrão do artigo original, dispensando o ítem RESULTADOS, máximo, máximo de 5 a 6 laudas.

Relato de caso

Deve ter relevância científica, conciso, máximo de 3 laudas, esquemático e didático; o método é o próprio relato do caso e dispensa resultados.

Nota prévia

Descrição de pesquisa inédita ou de inovação técnica, de maneira sucinta e objetiva,

máximo de 2 laudas.

Solicitamos aos autores e colaboradores da RPM que sigam as normas referidas e encaminhem os artigos após revisão e correção gramatical, inclusive o disquete.

No final de cada artigo, anotar o endereço completo com CEP, telefone para contato e endereço eletrônico (e-mail).

Endereço para correspondência

REVISTA PARAENSE DE MEDICINA
Fundação Santa Casa de Misericórdia do Pará
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Anexo C – Instruções para publicação no International Journal of Infectious Diseases

ELSEVIER

http://www.elsevier.com/wps/find/journaldescription.cws_home/701730/authorinstructions

International Journal of Infectious Diseases

Official Publication of the International Society for Infectious Diseases

Guide for Authors

If you have any problem submitting your paper online please contact Annette Fowler at IJID@elsevier.com

The *International Journal of Infectious Diseases* (IJID) is published monthly by the International Society for Infectious Diseases. *IJID*

Please noted as of January 2010 the International Journal of Infectious Diseases will be published online only:

Original articles on infectious disease topics of broad interest. We particularly welcome papers that discuss epidemiological aspects of international health, clinical reports, clinical trials and reports of laboratory investigations. Original articles should not exceed 5000 words in length.

Reviews on topics of importance to readers in diverse geographic areas. These should be comprehensive and fully referenced. Maximum length 6000 words.

Perspectives are papers that advance a hypothesis or represent an opinion relating to a topic of current interest or importance. They should be fully referenced, and should not exceed 2000 words in length.

Correspondence relating to papers recently published in the Journal, or containing brief reports of unusual or preliminary findings. Maximum length 400 words, one table or figure and a maximum of 10 references.

Case Reports must be carefully documented and must be of importance because they illustrate or describe unusual features or have important therapeutic implications. Maximum length 1200 words, no more than a page and a half in length and a maximum of 1 table or figure. Case reports do not require a structured abstract and should include no more than 5 references.

Medical Imagery: We would like to invite submission of high-quality, interesting and instructive images (such as clinical and other photographs, figures or diagrams, photomicrographs, or diagnostic imaging) suitable for the general readership of IJID. These should include no more than 200 words of explanatory text, and under 5 references. It is necessary to have appropriate permissions from subjects for an identifiable clinical image to be published.

Manuscript Submission

Manuscripts should be submitted online at: http://ees.elsevier.com/ijid

Covering letter: Manuscripts must be accompanied by a covering letter signed by ALL the authors stating that the current "Instructions to Authors" have been read, thereby indicating compliance with those instructions and acceptance of the conditions posed. The letter should state that the authors have seen and agreed to the submitted version of the paper, that all who have been acknowledged as contributors or as providers of personal communications have agreed to their

inclusion, that the material is original and that it has been neither published elsewhere nor submitted for publication simultaneously. In addition the letter should state that if accepted, the paper will not be published elsewhere in the same form, in English or in any other language, without written consent of the copyright holder.

A scanned image of the signed covering letter should be submitted via the online submission system. If this is not possible the letter should be posted or faxed to the Editorial Office.

It is strongly advised for Authors to suggest three non-conflicted peer reviewers with expertise as much for content as for methodology of their submission, with contact details including email address. This will significantly help the editorial office in facilitating timely external peer review.

Conflict of Interest: manuscripts cannot be published until we have received a signed Conflict of Interest Declaration form. You may obtain a Conflict of Interest Declaration document to be completed from the link below or upon request from the IJID Editorial Office IJID@elsevier.com. You are strongly advised to provide the declaration at the time you first submit your paper. A scanned image of the signed declaration should be submitted via the online submission system. If this is not possible the declaration should be posted or faxed to the Editorial Office.

Conflict of Interest Declaration (pdf format) [To read the PDF file you must have Adobe Acrobat Reader installed on your system. Download a free copy of Adobe Acrobat Reader. http://www.adobe.com/products/acrobat/readstep2.html]

Upon acceptance for publication, manuscripts will become the permanent property of the International Society for Infectious Diseases and may not be published elsewhere without the permission of the Society.

Manuscript format

General: The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. Do not embed 'graphically designed' equations or tables, but prepare these using the wordprocessor's facility.

When preparing tables, preferably use a table grid and use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts. Do not import the figures into the text file but instead, indicate their approximate locations directly in the electronic text. Each figure must be submitted separately as requested at the file upload stage of submission. For further information on the preparation of electronic illustrations, please refer to the "Tables and Figures" section.

To avoid unnecessary errors you are strongly advised to use the 'spellchecker' function of your wordprocessor.

The entire manuscript, including the abstract, acknowledgements, references, tables, figures, and legends, must be double spaced, with a margin of at least 2.5 cm. On assignment to an editor, each manuscript will be assigned a number, which will be provided to the author. The author should refer to this number in all ensuing correspondence. All manuscripts (including correspondence) will be subject to peer review. A rapid response to the authors will be more feasible if the manuscript is prepared as stipulated in the Instructions to Authors. Expressions of Latin origin, for example, in vivo, et al., per se should not be in italics.

Language and language services

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who require information about language editing and copyediting services pre- and post-submission please visit http://www.elsevier.com/languageediting or our customer support site at http://epsupport.elsevier.com for more information.

Numbers and measurements: Use decimal points (not commas); use a space for thousands (10 000 and above).

Title Page: The title page must include each author's full name and academic affiliations. The author to whom correspondence concerning the manuscript and to whom requests for reprints should be directed must be designated, as well as the corresponding address, telephone, fax, and e-mail. Manuscripts that were presented as part of a meeting must include the title, location, and date of the meeting on the title page. *Abstract*: A structured abstract of 150 to 200 words must be provided as part of each manuscript, except correspondence. The abstract should consist of four paragraphs, labelled with the following headings: objectives, design or methods, results, conclusions, or alternative headings appropriate to the format of the paper. The abstract should not refer to footnotes or references.

Keywords: Immediately after the abstract, provide a maximum of six keywords, avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be used.

Acknowledgements: Place acknowledgements, including information on grants received, before the references, in a separate section, and not as a footnote on the title page.

References: Indicate references by superscript numbers in the text.

Number the references in the list in the order in which they appear in the text.

Examples:

Reference to a journal publication:

1. Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2000;163:51-9.

Reference to a book:

2. Strunk Jr W, White EB. The elements of style. 3rd ed. New York: Macmillan; 1979. Reference to a chapter in an edited book:

3. Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 1999, p. 281-304.

Note shortened form for last page number. e.g., 51-9, and that for more than six authors the first six should be listed followed by 'et al.' For further details you are referred to "Uniform Requirements for Manuscripts submitted to Biomedical Journals" (J Am Med Assoc 1997;277:927-934) (see also http://www.nejm.org/general/text/requirements/1.htm) References to personal communications and to unpublished material must be incorporated, in parentheses, at the appropriate place in the text. References to congress abstracts should be cited in the reference section if they have been published previously in an official book of abstracts from the congress; otherwise they should be incorporated in the text. The author is responsible for the accuracy and completeness of the references.

Citing and listing of web references: Such article citations should include the DOI (digital object identifier).

For example:

Boutayeb A, Twizell EH, Achouayb K, Chetouani A. A mathematical model for the burden of diabetes and its complications. Biomed Eng Online 2004;3:20. doi:10.1186/1475-925X-3-20. The

DOI is a persistent identifier, which remains with the article even after it is published in print. See http://www.doi.org for details.

If the reference does not have a DOI, the full URL should be given. Any further information, if known (author names, dates, reference to a source publication, etc.), should also be given.

Style: For stylistic questions, authors are referred to the Chicago Manual of Style, 14th Edition, 1993, published by the University of Chicago Press.

Abbreviations: Abbreviations in the text are discouraged. If a term appears repeatedly, however, an abbreviation may be introduced parenthetically at the initial mention of the term and used thereafter in place of the term. Abbreviations of conventional or SI units of measurement may be used without introduction.

References to drugs: The generic name of a drug should be used as a general rule; however, the full name or the commercial name of the drug, as well as the name and location of the supplier, may be given in addition if appropriate.

Tables and Figures: Data reported either in a table or in a figure should be illustrative of information reported in the text, but should not be redundant with the text. Each table must be presented at the end of the manuscript on a separate page and numbered in order of appearance in the text. The title of the table must appear after the number. Each table must include appropriate headings. Footnotes, when necessary, must be identified by letters. Units of measurement must be clearly indicated.

Figures should not be imported into the manuscript text file but submitted separately as requested at the file upload stage of submission. A short detailed legend should be provided for each figure. All legends must be collected together on a separate page following the body of the manuscript.

For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Photomicrographs should include a micron bar or other appropriate scale marking.

Bacterial nomenclature: Microbes should be referred to by their scientific names according to the binomial system used in the latest edition of *Bergey's Manual of Systematic Bacteriology* (The Williams and Wilkins Co.). When first mentioned, the name should be in full and written in italics. Thereafter, the genus should be abbreviated to its initial letter, e.g. '*S. aureus*' not '*Staph. Aureus*'. If abbreviation is likely to cause confusion or render the intended meaning(s) unclear the names of organisms should be given in full. Only those names included in the Approved Lists of Bacterial Names (*Int J Syst Bacteriol* 1980; 30: 225-420) and/or which have been validly published in the *Int J Syst Bacteriol* since January 1980 are acceptable. If there is a good reason to use a name that does not have standing in nomenclature, it should be enclosed in quotation marks and an appropriate statement concerning its use made in the text (e.g. *Int J Syst Bacteriol* 1980; 30: 547-556).

Symbols for units of measurement must accord with the Système International (SI): However, blood pressure should be expressed in mmHg and haemoglobin as g/dl.

GenBank/DNA sequence linking: Many Elsevier journals cite "gene accession numbers" in their running text and footnotes. Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Elsevier authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should type this information in the following manner:

For *each* and *every* accession number cited in an article, authors should type the accession number in **bold, underlined text** . Letters in the accession number should always be capitalised. (See example below). This combination of letters and format will enable Elsevier's typesetters to recognise the relevant texts as accession numbers and add the required link to GenBank's sequences.

Example: "GenBank accession nos. **AI631510** , **AI631511** , **AI632198** , and **BF223228**), a B-

cell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank accession no. **AA361117**)".

Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.** In the final version of the ***printed*** **article**, the accession number text will not appear bold or underlined. In the final version of the ***electronic copy*** , the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article.

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Ethical Consideration. Work on human beings that is submitted to *International Journal of Infectious Diseases* should comply with the principles laid down in the Declaration of Helsinki; Recommendations guiding physicians in biomedical research involving human subjects. Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975, the 35th World Medical Assembly, Venice, Italy, October 1983, and the 41st World Medical Assembly, Hong Kong, September 1989. The manuscript should contain a statement that the work has been approved by the appropriate ethical committees related to the institution(s) in which it was performed and that subjects gave informed consent to the work. Studies involving experiments with animals must state that their care was in accordance with institution guidelines.

Studies on patients or volunteers require ethics committee approval and informed consent which should be documented in your paper. Patients have a right to privacy. Therefore identifying information, including patients¿ images, names, initials, or hospital numbers, should not be included in videos, recordings, written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and you have obtained written informed consent for publication in print and electronic form from the patient (or parent, guardian or next of kin where applicable). If such consent is made subject to any conditions, Elsevier must be made aware of all such conditions. Written consents must be provided to Elsevier on request. Even where consent has been given, identifying details should be omitted if they are not essential. If identifying characteristics are altered to protect anonymity, such as in genetic pedigrees, authors should provide assurance that alterations do not distort scientific meaning and editors should so note. If such consent has not been obtained, personal details of patients included in any part of the paper and in any supplementary materials (including all illustrations and videos) must be removed before submission.

Randomised Controlled Trials. All randomised controlled trials submitted for publication in *International Journal of Infectious Diseases* should include a completed Consolidated Standards of Reporting Trials (CONSORT) flow chart. Please refer to the CONSORT statement website at http://www.consort-statement.org/?0=1001 for more information. *International Journal of Infectious Diseases* has adopted the proposal from the International Committee of Medical Journal Editors (ICMJE) which require, as a condition of consideration for publication of clinical trials, registration in a public trials registry. Trials must register at or before the onset of patient enrolment. The clinical trial registration number should be included at the end of the abstract of

the article. For this purpose, a clinical trial is defined as any research project that prospectively assigns human subjects to intervention or comparison groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Studies designed for other purposes, such as to study pharmacokinetics or major toxicity (e.g. phase I trials) would be exempt. Further information can be found at www.icmje.org.

Conflicts of Interest. At the end of the text, under a subheading "Conflict of interest statement" all authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Authorship. All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Acknowledgement. All contributors who do not meet the criteria for authorship as defined above should be listed in an acknowledgements section. Examples of those who might be acknowledged include a person who provided purely technical help, writing assistance, or a department chair who provided only general support. Authors should disclose whether they had any writing assistance and identify the entity that paid for this assistance.

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• Authors can specify that they would like to select this option after receiving notification that their article has been accepted for publication, but not before. This eliminates a potential conflict of interest by ensuring that the journal does not have a financial incentive to accept an article for publication.

• Authors who have had their article accepted and who wish to sponsor their article to make it available to non-subscribers should complete and submit the sponsored article order form .

Anexo D - Instruções para publicação no Journal of Clinical Virology

ELSEVIER

http://www.elsevier.com/wps/find/journaldescription.cws_home/524062/authorinstructions

Journal of Clinical Virology

The Official journal of the Pan American Society for Clinical Virology and The European Society for Clinical Virology

Guide for Authors

Submission of an article to the ***Journal of Clinical Virology*** implies that the work described has not been published previously (except in the form of an Abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the Publisher.

Articles may only be submitted through the online submission system available at http://ees.elsevier.com/jcv. Authors submitting paper articles will be contacted and requested to submit online.

Please ensure that you have completed the following:

- Indicated the type of article submitted.
- Manuscript and abstract (according to the Guide for Authors) are complete and uploaded.
- **The structure of the manuscript and abstract is correct for each article type, see below.**
- All figures and tables have been uploaded and appear correctly in the pdf.
- All text pages, keywords, artwork, tables and figure captions have been included.
- Manuscript is spell-checked and within the word limit for the type of article.
- **If manuscript is over the word limit, approval from the Editor to exceed the word limit is obtained prior to submission. You will need to upload the Editor's approval when submitting the paper.**
- **Manuscripts should be submitted in double line spacing and the lines and pages should be numbered.**
- References are in the correct format for the journal. **Citations in the text are**

numbered superscripts.
- References mentioned in the reference list are cited in the text and vice versa.
- Permission has been obtained for use of copyrighted material from other sources (including the Web).
- Colour figures are clearly marked as being intended for colour or black-and-white reproduction.

Articles of the following types are accepted by the Editors.

Full-length Articles

Full-length articles should describe original research and/or clinical studies, and should be no more than 2,500 words excluding references and abstract. A structured Abstract of your study, of no more than 250 words, is required, and should include the following headings in bold: Background, Objectives, Study Design, Results, and Conclusions. The main text should be structured as follows: Background, Objectives, Study Design, Results, Discussion.

Short Communications

These articles should include a structured Abstract (see below), and be no longer than 1,250 words excluding references and abstract.A structured Abstract of your study, of no more than 250 words, is required, and should include the following headings in bold: Background, Objectives, Study Design, Results, and Conclusions. The main text should be structured as follows: Background, Objectives, Study Design, Results, Discussion.

Case Reports

These should be short articles of a clinical nature which illustrate or illuminate an underlying principle of a disease state, its diagnosis, or its therapy and should not exceed 1,500 words. All Case Reports should adhere to the following structure: **Why this case is important, Case description, other similar and contrasting cases in the literature, Discussion.**

My favourite assay The idea behind this is that it offers an opportunity for researchers to present an assay that they have developed that they feel would be useful for others. The articles should be a maximum of 2 printed pages long and are not peer reviewed. If others try the assay and it does not work, they are encouraged to submit a letter to the editor with their data. The Sections are: **Reasons for developing the assay, methods used in designing the assay, Protocol and Validation date** (dilution series, clinical samples, QCMD panels etc). Maximum 2 printed pages.

VIROQAS

Part I: **Case presentation.** Provide questions for readers at the end (bold).
Part II: **Evidence-based opinion.** Each sub-section should start with the question from Part I (in bold), and answer the question
Provide a page break between Part I and Part II.

Size limit:
Part I: maximum one printed page
Part II: maximum two printed pages
Word Limit: 1250

Letters to the Editor

Correspondence should be no more than 500 words. Comments on previously published articles or any other items of interest to clinical virologists may be considered.

Guidelines and Clinical Algorithms

These should be documents that have been locally or nationally approved and contain generalizable principles that may benefit others in their clinical practice or in writing their

own algorithms or guidelines.

Commentaries and Points of View

Please contact one of the Editors-in-Chief to check on the suitability of your topic. These types of article should be no more than 750 words.

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Prospective authors of review articles should contact one of the Editors-in-Chief prior to submission. These reviews should give a succinct overview of a particular topic. Review articles should be no more than 3,000 words.

Meeting Reports, News, and Announcements

Meeting reports should provide a summary of a relevant Symposium, Workshop, etc. and should not exceed 1,500 words. News or Announcements should be brief paragraphs about new developments within a relevant field and should not exceed 1,000 words.

Supplementary material

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Abstract
A structured Abstract of your study, of no more than 250 words, is required, for short communications and full length articles, and should include the following headings in bold: **Background, Objectives, Study Design, Results, and Conclusions.** Abstracts form the basis of electronic searches may influence a researcher's decision to read and cite your work, so it is very important that the abstract summarise your article fully and be able to stand alone to explain the work.

Key words
Key words (3-6) should be provided at the foot of the abstract.

Short communications and full-length articles should have the following sections in the manuscript:

Background
Objectives
Study Design
Results
Discussion
Acknowledgments and COI

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References

Please note the change in reference style for the journal from 1 April 2008. All papers submitted after 31 June 2008 in the 'old' reference style will need to be revised to the new format by authors

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Examples:

1. De Clercq E. Antiviral drugs: Current state of the art. J Clin Virol 2001;22,73-89.

2. Tartaglia J, Paoletti E. Live recombinant viral vaccines. In: van Regenmortel MHV and Neurath AR, editors. Immunochemistry of Viruses II. Amsterdam: Elsevier; 1990, p. 125-51.

3. Kaplan MH. Human herpesvirus-6 and HIV-1 infection. In: Ablashi DV, Krueger GRF, Salahuddin SZ, editors. Perspectives in Medical Virology, Vol. 4, Human herpesvirus-6. Amsterdam: Elsevier; 1992, p. 255-61.

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