FERNANDO CEZAR DOS SANTOS

POLIMORFISMOS INTRÔNICOS NO GENE DO FATOR DE TRANSCRIÇÃO FOXP3 E NÍVEIS DA IL-10 COMO BIOMARCADORES DE SUSCEPTIBILIDADE E PROGNÓSTICO NA ONCOGÊNESE CERVICAL INDUZIDA PELO HPV

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Orientadora: Profa. Dra. Karen Brajão de Oliveira

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Londrina, 8 de novembro de 2018.

A meu pai e minha mãe. Se pude sonhar
grande, foi por estar sobre seus ombros.

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Às **mulheres** que doaram voluntariamente as amostras biológicas para este estudo. Como disse o oncologista Siddhartha Mukherjee, no livro *O Imperador de Todos os Males: Uma Biografia do Câncer*, ‘‘o câncer não é uma doença, mas muitas’’. Essa constatação talvez seja a que faça mais sentido quando olhamos para o nosso delineamento, composto de tantas condições que partem de uma infecção primária. Essas mulheres são acometidas por essas ‘‘muitas’’ doenças devido a um complexo contexto que envolvem questões biológicas, sociais, políticas e bioéticas, que precisam ser abordadas com mais atenção. Trabalhar com diagnóstico de HPV é mais do que biologia e medicina, é um ato político, de denúncia social. É dizer: Estado, HPV MATA! Pessoas estão morrendo! Onde estamos falhando em mantê-las vivas?

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que as maiores especialistas no assunto deste trabalho serem elas, mas por reconhecer que finalmente, a hegemonia de gênero parece estar chegando ao fim, e agora a ciência é mais doce e gentil.

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A variabilidade é a lei da vida, e assim como dois rostos não são iguais, dois corpos também não são, e dois indivíduos não reagem nem se comportam da mesma forma quando submetidos a condições anormais que nós conhecemos como doença.

Sir William Osler

DOS SANTOS, Fernando Cezar. **Polimorfismos intrônicos no gene do fator de transcrição FOXP3 e níveis da IL-10 como biomarcadores de susceptibilidade e prognóstico na oncogênese cervical induzida pelo HPV.** 2018. 122 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2018.

RESUMO

A infecção persistente pelo papilomavírus humano (HPV) de alto risco (hrHPV) inicia e, em conjunto com outros fatores, promove a carcinogênese na cérvice uterina, a progressão para lesões intraepiteliais escamosas (SIL) e consequentemente para câncer de colo de útero (CCU). Ainda que o vírus seja bem-sucedido em causar infecção produtiva no hospedeiro, sua presença é notada pelo sistema imune, que é hábil em estabelecer respostas epiteliais de defesa eficientes e eliminar o vírus. No entanto, ao longo da evolução, o HPV desenvolveu a capacidade de se evadir da resposta imune, modulando a dinâmica imunológica, especialmente de células T, a seu favor. Alvo desses mecanismos de evasão são as células T regulatórias (Treg), identificadas pela expressão constitutiva do fator de transcrição *forkhead box* P3 (FOXP3). Estas células possuem atividade supressora sobre respostas efetoras e contribuem para a persistência da infecção e progressão tumoral. Neste contexto, variantes genéticas no gene *FOXP3* podem alterar seus níveis intracelulares e sua função, estando associadas à diversas doenças humanas. Desta forma, este estudo objetivou analisar os polimorfismos genéticos rs2232365 e rs3761548 em mulheres infectadas pelo HPV e portadoras de SIL e mulheres não infectadas livres de lesões e buscar uma possível correlação entre estes polimorfismos e os níveis plasmáticos e cervicais da interleucina 10 (IL-10). A detecção e genotipagem do HPV e genotipagem dos polimorfismos genéticos de *FOXP3* foram realizadas através da técnica de reação em cadeia da polimerase seguida de digestão enzimática (PCR RFLP). A dosagem da IL-10 foi realizada pelo ensaio imunoenzimático (ELISA) de captura. O genótipo homozigoto do polimorfismo rs3761548 (A/A) (relacionado à diminuição da expressão de *FOXP3*) é um bom preditor de proteção na infecção pelo HPV em mulheres (OR_{Aj} = 0.60; IC95% = 0.36 – 0.99; p = 0.049); este genótipo é também um preditor independente de proteção para o desenvolvimento de lesão intraepitelial de alto grau (HSIL) (OR_{Aj} = 0.28; IC95% = 0.11 – 0.68; p = 0.006). Além disso, o genótipo homozigoto (G/G) do polimorfismo rs2232365 (relacionado ao aumento da expressão de *FOXP3*) mostrou ser um fator de risco independente para a infecção por HPV (OR_{Aj} = 2.10; IC95% = 1.06 – 4.15; p = 0.033). A análise de haplótipos não mostrou associação significativa em nosso estudo. Além disso, não foram detectadas diferenças significativas entre os genótipos do *FOXP3* e os níveis de IL-10 em nosso estudo. Nós sugerimos que a presença de alelos poli- mórficos do gene *FOXP3* não afeta a expressão de IL-10. O presente estudo demonstrou, pela primeira vez, associações significativas entre variantes genéticas de *FOXP3* e a infecção por HPV e diagnóstico de SIL. Estes dados revelam que o uso dos polimorfismos de *FOXP3* podem ser úteis na prática clínica como marcadores moleculares de susceptibilidade e prognóstico à infecção pelo HPV e ao câncer de colo do útero, respectivamente.

Palavras-chave: HPV. SIL. FOXP3. Treg. IL-10. Polimorfismo genético.

DOS SANTOS, Fernando Cezar. **Intronic polymorphisms in the FOXP3 transcription factor gene and IL-10 levels as susceptibility and prognostic biomarkers of the HPV-induced cervical oncogenesis.** 2018**.** 122 p. Thesis (Doctoral in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2018.

ABSTRACT

Persistent infection by high-risk papillomavirus (hrHPV) initiates and, in conjunction with other factors, promotes carcinogenesis in the uterine cervix, progression to squamous intraepithelial lesions (SIL) and consequently cervical cancer (CC). Although the virus is successful in causing productive infection in the host, its presence is noted by the immune system, which is able to establish efficient defense epithelial responses and eliminate the virus. However, throughout evolution, HPV has developed the ability to evade the immune response, modulating immune dynamics, especially of T cells, in its favor. Targets of these evasion mechanisms are the regulatory T cells (Treg), identified by the constitutive expression of the transcription factor forkhead box P3 (FOXP3). These cells have suppressive activity on effector responses and contribute to the persistence of infection and tumor progression. In this context, genetic variants in the *FOXP3* gene may alter protein intracellular levels and its function, being associated with several human diseases. Thus, this study aimed to analyze the genetic polymorphisms rs2232365 and rs3761548 in women infected by HPV and carrying SIL and uninfected lesions-free women and to investigate a possible correlation between these polymorphisms and interleukin 10 (IL-10) plasma and cervical levels. HPV detection and genotyping and *FOXP3* genetic polymorphisms genotyping were performed using the PCR-RFLP (Restriction Fragment Length Polymorphism*)* technique. The IL-10 levels were performed by the capture immunoenzimatic assay (ELISA). The homozygous genotype of the rs3761548 (A/A) polymorphism (related to decreased expression of *FOXP3*) is a good predictor of protection in HPV infection in women (OR_{Adj} = 0.60; CI95% = 0.36 – 0.99; p = 0.049); this genotype is also an independent predictor of protection to the development of high-grade SIL (HSIL) (OR_{Adj} = 0.28; CI95% = 0.11 – 0.68; p = 0.006). In addition, the homozygous genotype (G/G) of the rs2232365 polymorphism (related to increased expression of *FOXP3*) has been shown to be an independent risk factor for HPV infection (OR_{Adj} = 2.10; CI95% = 1.06 – 4.15; p = 0.033). Haplotype analysis showed no significant association in our study. In addition, no significant differences were detected between *FOXP3* genotypes and IL-10 levels in our study. We suggest that the presence of polymorphic alleles of *FOXP3* gene does not affects IL-10 expression. The present study demonstrated for the first time significant associations between genetic variants of *FOXP3* and HPV infection and SIL diagnosis. These findings reveal the *FOXP3* polymorphisms may be useful in clinical practice as molecular markers of susceptibility and prognosis to HPV infection and cervical cancer, respectivelly.

Keywords: HPV. SIL. FOXP3. Treg. IL-10. Genetic polymorphisms.

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LISTA DE ABREVIATURAS E SIGLAS

aas	Aminoácidos
$AgNO_3$	Nitrato de prata
APC	Célula apresentadora de antígeno
ASC-H	Células escamosas atípicas não podendo excluir lesão intraepitelial de alto grau
ASC-US	Células escamosas atípicas de significado indeterminado
ATP	Adenosina trifosfato
CAAE	Certificado de Apresentação para Apreciação Ética
CCE	Carcinoma de células escamosas
CCU	Câncer de colo de útero
CD	Grupamento de diferenciação (*Cluster of differenciation)*
CEP	Comitê de Ética em Pesquisa
CIS	Carcinoma *in situ*
CNS	Sequência não codificadora
CSIF	Fator inibitório da síntese de citocinas
CTLA-4	Proteína 4 associada à linfócito T citotóxico (*Cytotoxic T-lymphocyte-associated protein 4)*
CTL	Linfócito T citotóxico
DC	Célula dendrítica
DM I	Diabetes mellitus do tipo I
DNA	Ácido desoxirribonucleico
dNTP	Desoxirribonucleotídeos fosfatados
E6AP	Proteína associada à proteína E6
EDTA	Ácido etilenodiaminotetracético
FOXP3	*Forkhead box* P3
H_2O	Água
HCl	Ácido Clorídrico
HGVS	Sociedade de Variação do Genoma Humano
HPV	Papilomavírus humano
hrHPV	Papilomavírus humano de alto risco oncogênico
HSIL	Lesão intraepitelial escamosa de alto grau
hTERT	Transcriptase reversa da telomerase

IARC	Agência Internacional de Pesquisa em Câncer
IC	Intervalo de confiança
IFN-ɣ	Interferon gama
IFN-α	Interferon alfa
IL	Interleucina
IL-10R	Receptor de interleucina 10
IL-12B	Subunidade beta da interleucina 12
IL-17RA	Receptor de interleucina 17 A
IL-2R	Receptor de interleucina 2
INCA	Instituto Nacional do Câncer
IQR	Interquartis
iTreg	Célula T regulatória induzível
JAK-STAT	Proteínas ativadoras de transcrição e transdutoras de sinal *Janus-Kinases* (*Janus kinases-Signal Transducer and Activator of Transcription proteins)*
JEC	Junção escamo-colunar
KCl	Cloreto de potássio
LAG3	Gene 3 de ativação de linfócito (*Lymphocyte-activation gene 3)*
LC	Célula de Langerhans
LCR	Região de controle longa (*Long control region)*
LES	Lúpus Eritematoso Sistêmico
lrHPV	Papilomavírus humano de baixo risco oncogênico
LSIL	Lesão intraepitelial escamosa de baixo grau
MAPK	Proteínas quinases ativadas por mitógeno (*Mitogen-activated protein kinases)*
$MgCl_2$	Cloreto de magnésio
miRNA	Micro RNA
NFAT	Fator nuclear de células T ativadas (*Nuclear factor of activated T-cells)*
NF-κB	Fator nuclear *kappa* B (*Factor nuclear kappa B)*
NIC	Neoplasia intraepitelial cervical
NK	Célula assassina natural (*natural killer)*
NKT	Célula assassina natural *natural killer* T
nTreg	Célula T regulatória natural
OFR	Quadro de leitura aberta (*Open Reading Frame)*
OR	Razão de chances (*Odds ratio)*

PAMP	Padrões moleculares associados à patógenos
Pap	Papanicolaou
pb	Par de base
PCR	Reação em cadeia da polimerase
pH	Potencial hidrogeniônico
PRR	Papilomatose respiratória recorrente
PRR	Receptores de reconhecimento de padrões PV Papilomavírus
RFLP	Polimorfismo no Comprimento do Fragmento de Restrição (*Restriction Fragment Length Polymorphism)*
RNA	Ácido ribonucleico
RORα	Receptor alfa orfão relacionado ao receptor de ácido retinóico (*Retinoid acid receptor-related orphan receptor alpha)*
SCID	Imunodeficiência combinada grave
SIL	Lesão intraepitelial escamosa
SMAD	*Small mothers against decapentaplegic protein*
SNP	Polimorfismo de nucleotídeo único
Sp1	Fator de transcrição Sp1
STAT	Transdutor de sinal e ativador de transcrição (*signal transducer and activator of transcription*)
TCLE	Termo de Consentimento Livre e Esclarecido TCR Receptor de célula T
TGF-β	Fator de crescimento transformante beta Th Célula T auxiliar (*helper)*
TLR	Receptores toll-*like*
TNF-α	Fator de necrose tumoral alfa Tr1 Células T regulatórias do tipo 1
Treg	Célula T regulatória
UEL	Universidade Estadual de Londrina
URR	Região regulatória a jusante (*Upstream Regulatory Region)*

SUMÁRIO

1 INTRODUÇÃO

A infecção pelo papilomavírus humano (HPV) é altamente comum entre populações humanas. Por todo o mundo, estudos de prevalência estimam que a média de prevalência da infecção pelo HPV entre mulheres é de 10,4%, embora entre mulheres com menos de 25 anos seja de 16,9% (CROSBIE et al., 2013). O câncer de colo de útero (CCU), que é caracterizado pela transformação maligna de células epiteliais cervicais após infecção persistente pelo HPV, é uma das neoplasias malignas mais comuns entre mulheres, representando quase 10% de todos os tumores ginecológicos. A cada ano, mais de 550.000 mulheres são diagnosticadas com CCU, principalmente em países em desenvolvimento, resultando em aproximadamente 311.000 mortes (BRAY et al., 2018).

Dados do Instituto Nacional do Câncer (INCA) apontam que, no Brasil, sem considerar os tumores de pele não melanoma, o CCU é o primeiro mais incidente na Região Norte (25,62/100 mil). Nas Regiões Nordeste (20,47/100 mil) e Centro-Oeste (18,32/100 mil), ocupa a segunda posição mais frequente; enquanto, nas Regiões Sul (14,07/100 mil) e Sudeste (9,97/100 mil), ocupa a quarta posição. Estimam-se 16.370 casos novos de CCU para cada ano do biênio 2018-2019, com um risco estimado de 15,43 casos a cada 100 mil mulheres, ocupando a terceira posição (INCA, 2017).

Estudos mostram que mais de 200 genótipos de HPV têm sido identificados até hoje, com novos tipos virais sendo constantemente descobertos (BZHALAVA; EKLUND; DILLNER, 2015). HPVs diferem em relação ao tropismo tecidual, sendo preferencialmente epiteliotrópicos e mucosotrópicos. Alguns tipos são patogênicos, levando a uma variedade de condições benignas (verrugas genitais e orais), bem como doenças malignas (as mais comuns sendo CCU, carcinomas penianos, vulvares, vaginais e esofágicos) (CAO et al., 2005; AL-DARAJI; SMITH, 2009).

HPVs tipos 16 e 18 são responsáveis por aproximadamente 60-80% de todos os casos de CCU, enquanto os tipos 52 e 31 causam a maioria dos casos remanescentes. No entanto, o padrão de distribuição do HPV difere significantemente entre diferentes populações (BRUNI et al., 2010). Exemplificando, se nos Estados Unidos o CCU é o décimo terceiro tipo de câncer mais incidente, em países subdesenvolvidos como a Angola, ele ocupa a primeira posição (BRAY et al., 2018). Essa variação é devido ao efeito combinado entre prevalência da infecção por HPV e qualidade dos programas de rastreamento, que explica em parte a correlação inversa entre a incidência do CCU e o nível socioeconômico do país (VACCARELLA et al., 2014).

O exame de Papanicolaou (Pap) é a principal estratégia usada em programas de rastreio para o diagnóstico de neoplasia intraepitelial cervical (NIC) e rastreamento e controle do CCU por todo o mundo. No entanto, a associação da citologia convencional e da biologia molecular pode elevar a sensibilidade e o valor preditivo negativo para cerca de 100%, sugerindo que mulheres com ambos os exames negativos possam ser submetidas a novo rastreamento em intervalos maiores. Por isso, alguns países tem adotado a testagem para HPV juntamente com o Pap para fornecer mais informações neste rastreamento (DERCHAIN; LONGATTO FILHO; SYRJANEN, 2005). Um exemplo recente é a implementação do Programa Nacional de Rastreamento Cervical pela Austrália, que combina a detecção molecular do DNA-HPV de 14 tipos oncogênicos associada à citologia em base líquida (HAWKES, 2018). É válido ponderar que a testagem não possui especificidade suficiente quando consideramos que apenas o fato de o indivíduo estar infectado não é suficiente para que ele desenvolva câncer.

Embora a infecção e colonização do epitélio cervical por HPV de alto risco oncogênico (hrHPV) sejam pré-requisitos para o desenvolvimento do CCU, a resposta imune local tem sido considerada um importante determinante na progressão e no desfecho clínico da doença (BOSCH; DE SANJOSÉ, 2007). A alta incidência de lesões displásicas associadas ao HPV em pacientes imunocomprometidos sustenta a hipótese que o sistema imunológico de fato pode influenciar na transformação de células epiteliais (BOSCH; DE SANJOSÉ, 2007). O caráter transitório da maioria das infecções pelo HPV e a regressão observada em certos tipos de NIC que regridem para um aspecto morfológico normal sugerem que há variabilidade na resposta imune local, em parte explicada pela influência exercida pelo *background* genético do hospedeiro (KANODIA; FAHEY; KAST, 2007).

Variações em genes que codificam mediadores imunes e inflamatórios tem sido mostradas como fatores importantes na susceptibilidade a uma grande variedade de distúrbios infeciosos e autoimunes, bem como na progressão tumoral (NETEA; WIJMENGA; O'NEILL, 2012; CERHAN et al., 2013). Polimorfismos de nucleotídeo único (SNPs) nos genes *IL-12B* e *IL-17RA*, importantes nas respostas celulares T_h1 e T_h17, respectivamente, estão associados à risco na oncogênese cervical e vulvar e atestam que a variabilidade genética participa na transformação epitelial neoplásica induzida pelo HPV (HARDIKAR et al., 2015).

Dentre as diversas variantes genéticas associadas à infecção pelo HPV e desenvolvimento de lesões precursoras do CCU descritas, investigar a ocorrência de SNPs no gene *FOXP3* e estabelecer uma possível relação com o vírus e os desdobramentos da infecção foram objetos de nosso interesse.

2 REFERENCIAL TEÓRICO

2.1 O PAPILOMAVÍRUS HUMANO (HPV)

Os papilomavírus (PVs) compreendem um grupo de vírus que infectam humanos e animais, e que tem coevoluído com seus hospedeiros com pouco fluxo gênico entre as espécies (BRAVO; SANJOSÉ; GOTTSCHLING, 2010). Em geral, o resultado desta coevolução são infecções crônicas inaparentes e não doença severa, haja vista que HPVs são isolados de *swabs* cutâneos de indivíduos imunocompetentes, sugerindo que existe de fato comensalismo (ANTONSSON et al., 2000).

No entanto, apesar destas infecções de caráter não patogênico, o HPV pode causar doença severa e progressiva. O CCU é a mais significante delas, resultante da infecção persistente por um grupo de hrHPVs (DOORBAR et al., 2012). HPVs de baixo risco (lrHPV) não são comumente associados com o desenvolvimento de câncer, mas podem causar doenças igualmente incapacitantes, como é o caso da papilomatose respiratória recorrente (PRR), doença rara associada à infecção por HPV 11 que provoca o aparecimento de papilomas recorrentes nas vias aéreas e demanda cirurgias repetidas (GOON et al., 2008).

HPVs são divididos dentro de cinco gêneros baseado em análise de sequências de DNA, apresentando características de ciclo de vida e associações a doenças diferentes. PVs Beta e Gama possuem tropismo epitelial e causam infecções assintomáticas em indivíduos imunocompetentes, indicando que existe um equilíbrio entre replicação e imunotolerância. PVs Mu e Nu são associados às lesões cutâneas benignas; PVs PVs são tanto epitélio- quanto mucosotrópicos e incluem tanto hrHPV associados ao desenvolvimento do CCU quanto lrHPVs relacionados ao aparecimento dos condilomas benignos (verrugas genitais) (DOORBAR et al., 2012).

Tabela 1. Prevalência de HPV tipo-específica no CCU invasivo no mundo.

	Invasive cervical cancer			Normal		
	N tested	% pos	95%CI	N tested	% pos	95%CI
HPV 16	14595	54.4	53.6–55.2	76385	2.6	2.5–2.8
HPV 18	14387	15.9	15.3–16.5	76385	0.9	0.8–1.0
HPV 33	13827	4.3	4.0–4.6	74141	0.5	0.4–0.5
HPV 45	9843	3.7	3.3–4.1	65806	0.4	0.4–0.4
HPV 31	11960	3.5	3.2–3.9	74076	0.6	0.6–0.7
HPV 58	10157	3.3	2.9–3.6	72877	0.9	0.8–1.0
HPV 52	9509	2.5	2.2–2.8	69030	0.9	0.8–1.0
HPV 35	9507	1.7	1.5–2.0	74084	0.4	0.3–0.4
HPV 59	13471	1.28	1.09–1.47	64901	0.3	0.2–0.3
HPV 51	13057	1.16	0.97–1.34	67139	0.6	0.6–0.7
HPV 56	13247	0.78	0.63–0.93	68121	0.5	0.5–0.6
HPV 39	13370	1.29	1.10–1.48	64521	0.4	0.3–0.4
HPV 68	11982	0.61	0.47–0.75	63210	0.3	0.2–0.3
HPV 73	9939	0.48	0.35–0.62	44063	0.1	0.1–0.1
HPV 66	12118	0.39	0.28–0.50	59774	0.4	0.3–0.4
HPV 70	10503	0.33	0.22–0.44	35014	0.3	0.3–0.3
HPV 82	9265	0.27	0.16–0.38	42536	0.1	0.0–0.1
HPV 26	6111	0.13	0.04–0.22	44098	0.0	0.0–0.1
HPV 53	8140	0.42	0.28–0.56	44058	0.4	0.4–0.4
HPV 6	14912	0.45	0.35–0.56	58370	0.3	0.2–0.3
HPV 11	8761	0.2	0.1–0.4	58370	0.2	0.2–0.2

Fonte: IARC Monograph 100B (2012).

PVs Alfa mucosotrópicos são os tipos virais relativamente melhores entendidos, especialmente o HPV 16, o tipo mais prevalente relacionado ao CCU invasivo (Tabela 1). Apesar da biologia do processo infeccioso mediado por esse tipo na ectocérvix ser bem conhecido, seu ciclo de vida em outros sítios anatômicos ainda merecem uma melhor compreensão.

2.2 Estrutura Viral e Organização Genômica

PVs são pequenos, com um capsídeo icosaédrico não envelopado, e um genoma viral que consiste em um episssoma de DNA circular dupla fita com aproximadamente 8 kb. Genomas virais geralmente contêm uma região regulatória designada URR (*upstream regulatory region*) ou LCR (*long control region*), que contém sítios de ligação para fatores de transcrição e o sítio de início da transcrição, e nove ORFs (*open reading frames*), designadas de precoces (E) ou tardias (L) (Figura 1). Estes genes nucleares incluem as proteínas E1 e E2, necessárias para a regulação da replicação do DNA e transcrição, respectivamente, e proteínas virais tardias L1 e L2, que compõem a estrutura do capsídeo. L1 é a principal proteína do capsídeo, mais abundante. L2 está presente em menor número, e está envolvida na encapsulação do DNA viral e na facilitação da sua entrada e tráfego ao núcleo. Os genes virais restantes estão

envolvidos na liberação de partículas virais e transformação celular (E4, E5, E6 e E7) (MORSHED et al., 2014; EGAWA et al., 2015).

E1 atua na replicação através da sua atividade de helicase dependente de adenosina trifosfato (ATP). E2 é um coativador da replicação genômica viral através do recrutamento de E1 ao sítio de iniciação da replicação viral. E4 é expressa em estágios tardios do ciclo de vida do vírus e liga-se a filamentos de citoqueratina, causando sua ruptura, contribuindo com a liberação e transmissão do vírus (EGAWA et al., 2015). Portanto, L1, L2, E1, E2 e E4 são consideradas proteínas nucleares e E5, E6 e E7 proteínas acessórias.

A classificação dos PVs é baseada em comparação de sequências nucleotídicas a partir do gene referência *L1*, a ORF mais conservada dentro do genoma. Para ser classificado como um tipo distinto, PVs individuais devem ser pelo menos 10% divergentes um do outro na sequência nucleotídica de L1 (DE VILLIERS et al., 2004).

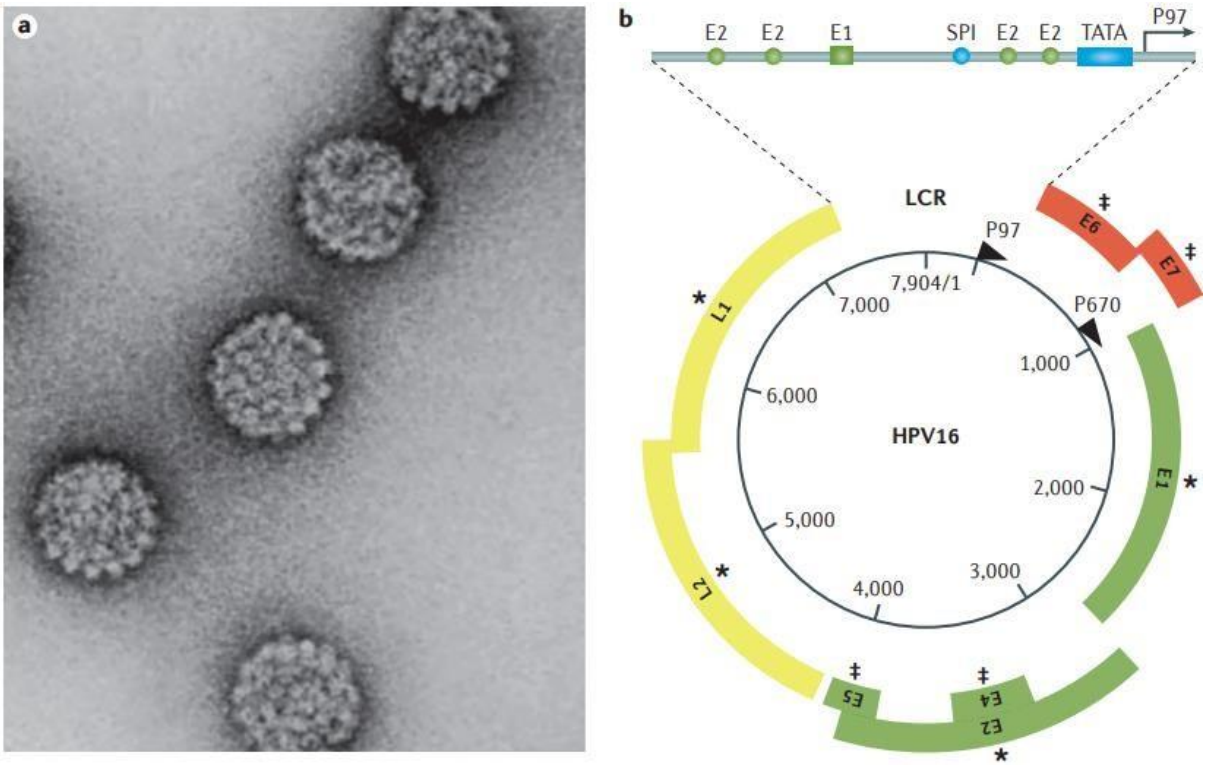

Figura 1. Estrutura do HPV e organização genômica.

a) Partículas virais do HPV (55 nm de diâmetro) são visualizadas por microscopia eletrônica de transmissão. **b**) A organização genômica típica de um HPV de alto-risco do gênero alfa, ilustrado como o HPV 16. Promotores precoce (P97) e tardio (P670) são indicados por pontas de seta. As seis ORFs precoces E1, E2, E4 e E5 (em verde) e E6 e E7 (em vermelho), são expressas a partir de diferentes promotores e diferentes estágios durante a diferenciação celular epitelial. As ORFs tardias L1 e L2 (em

amarelo) são expressas pelo promotor P670 nas camadas epiteliais superiores como resultado em mudanças no processo de *splicing*. A região de controle longa (LCR, as vezes referida como URR) é uma região não codificante do genoma que contém o sítio de origem da replicação bem como sequências de controle pós-transcricionais que contribuem para a expressão gênica. Os sítios de ligação para as proteínas virais E1 e E2 e para o fator de transcrição Sp1 são mostrados. Os produtos dos genes indicados com um asterisco (*) são proteínas nucleares necessárias para a replicação do genoma, montagem viral e liberação; produtos gênicos indicados com símbolo de diferente (≠) são proteínas acessórias com funções que incluem entrada no ciclo celular e evasão imune.
Fonte: SCHIFFMAN et al. (2016)

2.3 Ciclo de Vida

HPVs são patógenos exclusivamente intraepiteliais, e a infecção e o crescimento viral vegetativo são absolutamente dependentes da expressão do programa completo de diferenciação dos queratinócitos. Acredita-se que o vírus infecta queratinócitos basais e parabasais expostos que provavelmente possuem um fenótipo de célula-tronco durante o processo de regeneração. No entanto, alta taxa de expressão gênica/proteica e montagem do vírus ocorrem somente nas camadas superficiais bem diferenciadas do epitélio escamoso (MORSHED et al., 2014).

Após infectar o queratinócito, parece existir um *round* de replicação viral que independe do ciclo celular e amplifica o número de cópias virais em cerca de 50 a 100 cópias por célula. O queratinócito deixa então essa camada primitiva e passa a migrar para outro compartimento proliferativo transitório do epitélio, onde existe uma fase de manutenção plasmidial ou episomal, quando o número de cópias virais permanece constante e a expressão gênica é mínima. Nesta fase, especialmente em hrHPV, a expressão dos oncogenes *E6* e *E7* sofre um rigoroso controle. Quando o queratinócito entra no compartimento de diferenciação na camada espinhosa, onde as células saíram do ciclo celular, existe uma regulação positiva massiva da expressão gênica e replicação viral, com amplificação do número de cópias virais para milhares por célula (CHOW; BROKER; STEINBERG, 2010).

O que permite o acesso do HPV à lâmina basal são lesões ou micro-lesões epiteliais, e o papel da resposta fisiológica de regeneração celular em estimular a expansão do vírus tem sido sugerido (Figura 1) (SCHILLER; DAY; KINES, 2010). De fato, divisão celular ativa, como ocorreria durante o processo de reparo das lesões, parece ser necessária para a entrada do genoma viral dentro do núcleo, e tem sido proposto que a formação de lesões requer a

infecção inicial de células mitoticamente ativas. É importante lembrar que HPVs codificam apenas uma enzima envolvida na replicação celular, E1. Exceto pela ação de E1 e E2, a replicação é totalmente dependente da maquinaria biosintética celular. Assim, o vírus se aproveita de DNA polimerases celulares e outros fatores de replicação que são produzidos somente em células mitoticamente ativas (PYEON et al., 2009).

Modelos experimentais sugerem que para que a infecção ocorra as partículas virais (compostas de DNA viral e L1/L2) precisam ter acesso à lâmina basal e interagirem com heparan sulfato proteoglicanos (HSPGs) e laminina 5 (CULP et al., 2006; JOHNSON et al., 2009). Mudanças conformacionais no capsídeo do vírion, que inclui clivagem da porção N-terminal de L2 pela protease furina, induzem a exposição de L1 e facilitam a transferência para um receptor secundário ainda elusivo no queratinócito basal, que é necessário para a internalização do vírus e subsequente transferência do genoma viral para o núcleo. Embora integrinas e receptores de fatores de crescimento tem sido implicados neste processo, a natureza precisa do receptor de entrada permanece controversa. Uma vez internalizado, vírions sofrem transporte endossomal, perdem seu revestimento no endossomo tardio e escapam do endossomo por um mecanismo dependente de L2. O complexo L2-DNA é responsável pela entrada nuclear correta, enquanto a proteína L1 é retida no endossomo e sujeita a degradação lisossomal. Uma vez no núcleo, o complexo L2-DNA inicia o processo de transcrição gênica viral (SCHILLER; DAY; KINES, 2010).

hrHPVs levam à tumorigênese na junção escamo-colunar (JEC). Devido à anatomia cervical e o *status* hormonal, a posição da JEC varia. Na maioria das mulheres, a acidificação do pH vaginal que ocorre na adolescência induz a substituição de uma porção do epitélio colunar endocervical por um epitélio escamoso metaplásico. Esta área de substituição (zona de transformação) leva a migração proximal da JEC (HERFS et al., 2012).

A susceptibilidade particular da zona de transformação em predispor a progressão do câncer pode também estar ligada à acessibilidade aumentada e a proliferação das camadas celulares basais neste sítio epitelial metaplásico, particularmente na puberdade e no início da atividade sexual (GRAYSON et al., 2002) (Figura 2). Neste caso, hipotetiza-se que os alvos celulares primários para a infecção podem ser células perto da JEC como as células epiteliais de reserva, que estão presentes imediatamente subjacentes ao epitélio colunar da endocérvice, e que eventualmente formam as camadas epiteliais estratificadas a partir da zona de transformação para a cérvice madura (MARTENS et al., 2009). Uma pequena população de células localizadas na JEC descritas como ‘‘células juncionais’’ também podem ser alvos da infecção por HPV e estão implicadas no desenvolvimento do CCU (HERFS et al., 2012).

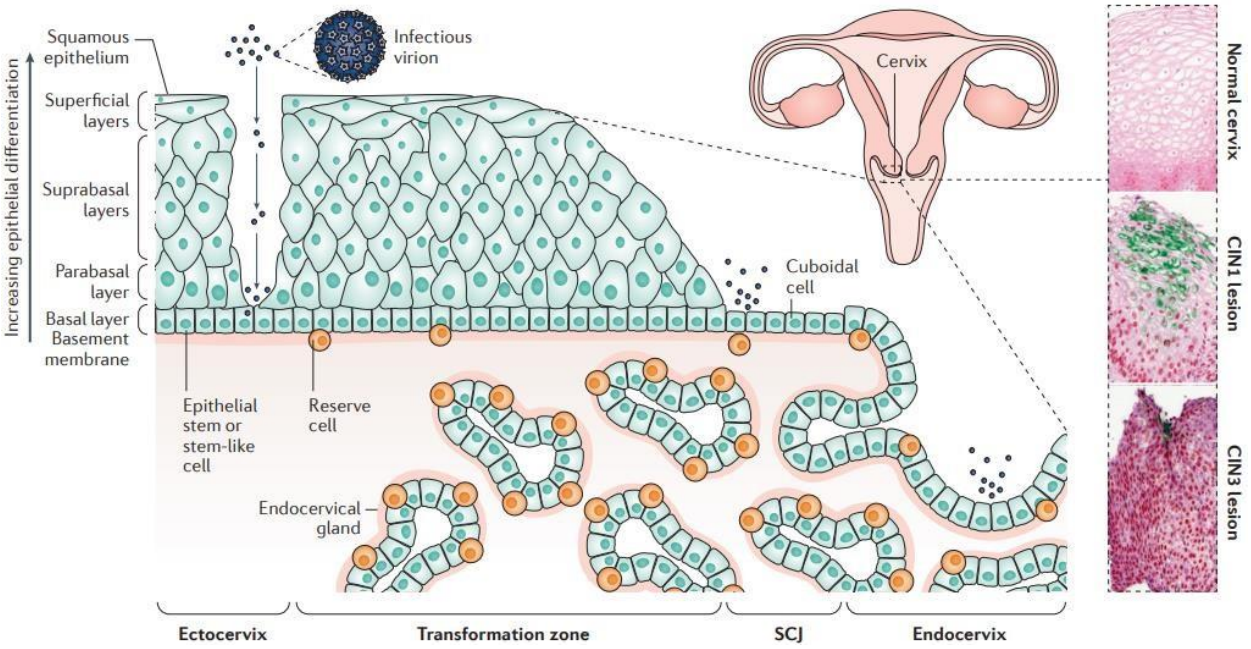

Figura 2. Infecção pelo HPV e a zona de transformação.

A maioria dos cânceres na cérvice uterina surgem na zona de transformação e na endocérvice adjacente, composta inicialmente de epitélio colunar que sofre metaplasia (particularmente na puberdade) para formar uma camada epitelial escamosa totalmente diferenciada. Camadas estratificadas da ectocérvice são mantidas por células-tronco epiteliais localizadas na camada basal. Em contraste, camadas estratificadas da zona de transformação e a camada única de células colunares que revestem a endocérvice são mantidas por células de reserva. Embora células de reserva sejam abundantes em regiões metaplásicas, células semelhantes às células-tronco com uma aparência cuboide localizadas mais precisamente na junção entre os epitélios (JEC) (e por isso denominadas ‘‘juncionais’’) também estão envolvidas na transformação celular. Hipotetiza-se que uma infecção produtiva por hrHPV é favorecida na JEC e regiões metaplásicas e que a formação da lesão começa da infecção de células-tronco epiteliais (de reserva ou cuboides) na zona de transformação ou endocérvice. Imagens a direita demonstram a cérvice normal e lesões estadiadas como neoplasia intraepitelial cervical (NIC) 1 e NIC3 imunomarcadas para proteína viral E4 e um complexo proteico marcador da entrada do vírus no ciclo celular (verde e vermelho, respectivamente).

Fonte: SCHIFFMAN et al. (2016)

2.4 Carcinogênese Viral

O HPV induz proliferação de células basais e parabasais, o que leva à displasia e/ou papilomatose com diversas extensões. Essa oncogenicidade é devida à atividade das

proteínas virais E5, E6 e E7. Estas oncoproteínas provocam proliferação celular contínua e uma inabilidade da célula em reparar possíveis danos no seu material genético, acumulando rearranjos, aneuploidias e mutações que levam ao desenvolvimento do câncer. Diversas rotas moleculares e bioquímicas celulares são alteradas, como as que estão envolvidas em processos de divisão e apoptose (MCLAUGHLIN-DRUBIN; MÜNGER, 2009).

A proteína E7 é conhecida por se ligar a supressores tumorais da família pRb, levando à sua degradação e ativação descontrolada do fator de transcrição E2F, que estimula a expressão de genes envolvidos na progressão para a fase S do ciclo celular (DYSON et al., 1989). Além disso, E7 desregula o controle do ciclo celular inativando inibidores de quinases dependentes de ciclinas (como p21 e p27) e estimulando a atividade das ciclinas através da ativação da quinase dependente de ciclina 2 (CDK2). E7 estimula a síntese anormal de centrossomos através da atividade aumentada de CDK2, levando a um aumento do risco de instabilidade genômica (MOODY; LAIMINS, 2010).

A proteína E6 também induz a inativação proteolítica de proteínas pró-apoptóticas como Bak, Bax, c-Myc e p53, possibilitando a manutenção de um fenótipo neoplásico. De fato, o desenvolvimento de instabilidade genômica é um *hallmark* de tumores associados ao HPV, e se deve principalmente à hipoatividade da p53 em reparar erros de replicação e induzir apoptose (DUENSING; MÜNGER, 2002; FINZER; AGUILAR-LEMARROY; RÖSL, 2002). E6 recruta a ubiquitina ligase E6AP, necessária para manter sua estabilidade, e o complexo E6/E6AP endereça a p53 poliubiquitinada para a degradação proteica mediada pelo barril proteassomal (THATTE; BANKS, 2017). E6 também é responsável por induzir a ativação da telomerase e da região promotora do gene da transcriptase reversa da telomerase (*hTERT),* fatores chaves na manutenção do comprimento dos telômeros e imortalização celular em um contexto tumoral (PAŃCZYSZYN; BONIEWSKA-BERNACKA; GŁĄB, 2018).

A integração de sequências do HPV dentro do genoma humano é considerada um evento crucial na progressão de tumores cervicais. Este passo ocorre através da ruptura do sítio de clivagem dos genes *E1/E2*, causando a ruptura do gene *E2* e regiões adjacentes de *E4, E5 e L2* (UEDA et al., 2003). Assim, a repressão transcricional exercida por E2 é anulada, resultando na superexpressão de *E6 e E7* e doença maligna severa (MUÑOZ et al., 2006).

Embora essas oncoproteínas tenham crucial importância nas propriedades transformadoras do HPV, o papel da proteína E5 no desenvolvimento do câncer tem sido cada vez mais explorado. As atividades desta oncoproteína suportam a progressão tumoral, particularmente nos estágios iniciais da doença, uma vez que o gene *E5* é deletado após o DNA viral ser integrado no genoma humano (DIMAIO; MATTOON, 2001). Existem muitos mecanismos

nos quais E5 está incluída que envolvem várias vias de sinalização de proliferação celular, angiogênese e apoptose. Uma das mais bem conhecidas atividades tumorigênicas mediadas por E5 é sua interação com o receptor do fator de crescimento epidérmico (EGFR), que leva à proliferação celular (OH et al., 2009; PEDROZA-SAAVEDRA et al., 2010).

Outro mecanismo associado com a carcinogênese induzida pelo HPV é a modulação de micro RNAs (miRNAs) do hospedeiro. A interferência do HPV na expressão de miRNAs é causada pelo fato que mais de 50% dos genes de miRNA estão localizados em sítios frágeis de integração de hrHPV (CALIN et al., 2004). A integração pode alterar a expressão de miRNA via deleção, amplificação, ou rearranjo genômico. No entanto, alguns estudos funcionais têm revelado que perfis aberrantes de alguns miRNAs são devido às atividades das oncoproteínas E5, E6 e E7 (GRECO et al., 2011; ZHENG; WANG, 2011).

2.5 Anormalidades Citológicas e o Carcinoma cervical

A coilocitose é a alteração celular patognomônica de replicação viral e sugere efeito citopático do HPV nas células mais superficiais do epitélio metaplásico e na JEC. Os principais critérios citológicos e histológicos são: aumento acentuado dos núcleos, irregularidade da membrana nuclear, distribuição irregular da cromatina com granulações grosseiras, hipercromasia nuclear, halo perinuclear fortemente demarcado com espessamento da membrana celular e binucleações (CARVALHO, 2012).

O epitélio endocervical não apresenta alterações coilocitóticas características que são frequentemente vistas na ectocérvice. Lesões de baixo grau, se presentes, localizam-se em áreas de metaplasia escamosa, onde se observa características comuns às encontradas na ectocérvice como: cariomegalia, halo perinuclear, hipercromatismo, binucleação. Porém, quando o comprometimento glandular é significativo, pode representar extensão direta de lesão de alto grau presente na ectocérvice adjacente (CARVALHO, 2012).

Alterações celulares leves ou inconclusivas são designadas como ASCUS (células escamosas atípicas de significado indeterminado) (GUTMAN et al., 2004). Lesões precursoras do CCU são denominadas neoplasia intraepithelial cervical (NIC) ou lesão intraepitelial escamosa (SIL), de acordo com os sistemas Richart e Bethesda, respectivamente. No sistema Richart, NIC 1 corresponde à displasia leve e NIC2 à displasia moderada. NIC 3 compreende displasia severa e carcinoma *in situ* (CIS) (SELVAGGI, 2015). No intuito de facilitar a interpretação de dados clinicamente relevantes, a Nomenclatura Bethesda para Citologia Cervicovaginal agrupou esta terminologia em lesão intraepitelial escamosa de baixo grau (LSIL)

(i.e., NIC 1) e lesão intrapitelial escamosa de alto grau (HSIL) (i.e., NIC 2/3 e CIS) (SOLOMON et al., 2002; NAYAR; WILBUR, 2015).

É importante destacar que as alterações inicialmente classificadas como ASCUS foram subdivididas no Sistema Bethesda em células escamosas atípicas de significado indeterminado (ASC-US) e células escamosas atípicas não podendo excluir lesão intraepitelial de alto grau (ASC-H). Essa nova classificação foi uma tentativa de refletir melhor as alterações que, apesar de menos definidas, poderiam representar lesões precursoras do CCU (FERNANDES et al., 2012).

O carcinoma de células escamosas (CCE) é o subtipo histológico mais comum de CCU (80% dos casos), seguido pelo adenocarcinoma cervical (15%) e por carcinomas mais raros, os adenoescamosos e neuroendócrinos (5%). O CCU invasivo pode apresentar crescimento exofítico ou infiltrativo. Ao exame histológico, o CCE é composto por ninhos e projeções de epitélio escamoso maligno, queratinizado ou não queratinizado, invadindo o estroma cervical subjacente. Os adenocarcinomas são caracterizados pela proliferação de epitélio glandular composto por células endocervicais malignas com núcleos grandes, hipercromáticos e citoplasma relativamente depletado de mucina, resultando em um aspecto escuro das glândulas, em comparação ao epitélio endocervical normal. O CCU avançado pode disseminar-se para tecidos paracervicais, a bexiga urinária, os ureteres, o reto e a vagina. Linfonodos locais e distantes também são envolvidos. Metástases distantes podem ser encontradas no fígado, pulmões, medula óssea e outras estruturas (KUMAR; ABBAS; ASTER, 2015).

2.6 A Resposta Imune Anti-HPV

A infecção pelo HPV é completamente dependente do programa de diferenciação dos queratinócitos, uma célula que naturalmente morre e descama longe de sítios de atividade imune. O ciclo infeccioso do vírus leva tempo, desde a infecção até sua liberação, e dura pelo menos três semanas, tempo necessário para o queratinócito basal migrar ao longo do epitélio e descamar. O período entre infecção e o aparecimento de lesões é variável e leva de semanas a meses, sugerindo que o vírus de fato se evade da resposta imune. O HPV não induz morte celular, e consequentemente não induz inflamação, e durante seu ciclo celular existe pouca liberação de citocinas pró-inflamatórias, importante para a ativação de células apresentadoras de antígenos (APCs) no microambiente. Ele é exclusivamente intraepitelial, não causa viremia, e pouca quantidade de partículas virais são expostas às defesas imunes (STANLEY, 2006).

A habilidade do hrHPV em comprometer o papel dos queratinócitos como sentinelas da imunidade inata é de suma importância para que a infecção seja bem-sucedida. Essas células respondem à lesão e estresse celular e são capazes de detectar patógenos (NESTLE et al., 2009). Essa capacidade é mediada principalmente pela expressão de receptores de reconhecimento de padrões (PRRs), que reconhecem *motifs* invariantes conhecidos como padrões moleculares associados à patógenos (PAMPs). A classe de PRRs melhor estudada são os receptores *Toll-like* (TLRs), e ativação dos TLRs desencadeia vias de sinalização que medeiam respostas inatas e adaptativas. Queratinócitos do trato genital expressam vários TLRs, localizados na superfície celular (TLR1, TLR2, TLR4, TLR5 e TLR6) ou endossomais (TLR3, TLR7, TLR8 e TLR9). Crucialmente, ativação de TLRs em queratinócitos leva à produção de interferons (IFNs) do tipo I (e.g., IFN-α), conhecidamente antivirais e antitumorais, e respostas citotóxicas padrão T *helper* (T_h) 1, protetoras em infecções virais (NESTLE et al., 2009).

Os principais TLRs responsáveis por reconhecer ácidos nucleicos virais (TLR3, TLR7, TLR8, TLR9) e ativar a transcrição mediada pelo NF-κB de genes pró-inflamatórios estão regulados positivamente e envolvidos na eliminação do HPV. No entanto, infecção persistente parece estar relacionada com a capacidade das oncoproteínas virais E6 e E7 em modular negativamente vias de sinalização mediadas por TLR9 (ZHOU; ZHU; CHENG, 2013). Uma vez que o HPV interage com TLRs expressos na superfície celular, estes receptores podem desempenhar um papel na entrada do vírus na célula. No entanto, não existem estudos demonstrando essa interação.

hrHPVs regulam negativamente a expressão de genes induzíveis por IFN-α, e oncoproteínas E6 e E7 do HPV16 interagem diretamente com componentes das vias de sinalização de IFNs, especialmente vias JAK-STAT, inibindo sua transcrição (BARNARD; MCMILLAN, 1999; JAIN et al., 2006). Além disso, hrHPVs episssomais são capazes de inibir uma gama de citocinas e quimiocinas pró-inflamatórias e moléculas apresentadoras/processadoras de antígenos, bem como a montagem da plataforma molecular inflamassoma, responsável pela maturação da interleucina (IL) 1β (IL-1β), que possui um papel chave em queratinócitos por induzir a ativação de células T_h, células dendríticas e células B (KARIM et al., 2011).

Apesar de o HPV induzir um estado de imunotolerância com pouca ou nenhuma inflamação, eventualmente ele é detectado pelo sistema imune, e a capacidade de eliminar a infecção reflete a montagem de uma resposta imune eficiente em estágios iniciais da entrada do HPV. Prova disso é que pelo menos 80 a 90% das infecções genitais pelo HPV são eliminadas com o tempo (MOSCIKI et al., 2012). Woodman, Collins e Young (2007) apontam que a mediana da duração de uma infecção persistente é de 6 meses, em um intervalo variando

de 2 meses a 7 anos. Estes autores também destacam que uma infecção é definida como persistente quando a testagem para o HPV é positiva pelo menos duas vezes, e que estudos que utilizam este termo usam intervalos de testagem distintos, levando à inferência de que a duração de uma infecção persistente não é uma constante.

Nos estágios iniciais da infecção pelo HPV, a resposta imune inata envolvendo macrófagos, células dendríticas (DCs), *natural killer* (NK), e *natural killer* T (NKT), bem como os queratinócitos, é a primeira linha de defesa contra a infecção. Como segunda linha de defesa, a imunidade adaptativa elimina células infectadas e previne a reinfecção, produzindo uma resposta de células T citotóxicas específica, com linfócitos t citotóxicos (CTLs) combatendo proteínas virais, como E2 e E6 (AMADOR-MOLINA et al., 2013). Apesar de estarem limitados pelos mecanismos de escape induzidos pelo HPV, os queratinócitos atuam como APCs não profissionais, induzindo a expressão de citocinas T_h1 e T_h2 em células T $CD4^+$, bem como resposta citotóxica de células T $CD8^+$ (BLACK et al., 2007).

Apesar da ação de queratinócitos, macrófagos e DCs são responsáveis por conectar respostas inatas e adaptativas. Macrófagos são ativados por componentes virais através de TLRs, liberando citocinas e quimiocinas pró-inflamatórias e INF-ɣ, levando a eliminação da célula infectada pelo HPV via secreção do fator de necrose tumoral alfa (TNF-α) ou citotoxicidade dependente de anticorpos. Queratinócitos secretam proteína quimiotática de monócitos 1 (MCP-1) na presença de TNF-α, atraindo mais macrófagos para o sítio de infecção. DCs, macrófagos e células B localizados na derme tem acesso a proteínas do HPV, mas eventualmente, devido às microlesões induzidas por infecções bacterianas ou contato sexual, essas proteínas são expostas e reconhecidas por células de Langerhans (LCs) na epiderme e respostas adaptativas iniciam, produzindo inflamação local e ativação de CTLs. Na presença de citocinas T_h1, como IL-2, IL-12 e INF-ɣ, CTLs se tornam células efetoras ativadas hábeis em destruir células cervicais pré-neoplásicas ou expressando antígenos do HPV (SASAGAWA; TAKAGI; MAKINODA, 2012). No entanto, estes mecanismos podem ser prejudicados se uma determinada população de células T com capacidade de induzir tolerância imunológica estiver presente no sítio infeccioso, a célula T regulatória.

2.7 Células T Regulatórias

A existência de células T regulatórias (Treg) foi postulada pela primeira vez no final da década de 1960 por Nishizuka e Sakakura, e no começo da década de 1970 por

Gershon e Kondo. Essa distinta população de células T foi originalmente caracterizada em camundongos, representando 5-10% de células T $CD4^+$ periféricas (KACZOROWSKI; JUTEL, 2013). O papel destas células é essencial em induzir tolerância imunológica central e periférica, e a desregulação imune relacionada a uma super-reação a estruturas próprias — evento que nós conhecemos como autoimunidade — pode ser explicada por defeitos na função de células Tregs. Sabe-se que números reduzidos e/ou disfunção de Tregs estão associados a doenças autoimunes como lúpus eritematoso sistêmico (LES), diabetes mellitus do tipo I (DMI) e imunodeficiência combinada grave (SCID) (PASSERINI et al., 2014).

Em humanos, células Treg são uma pequena subpopulação de linfócitos T $CD4^+$ (cerca de 5%) e são caracterizadas pela expressão do fator de transcrição *forkhead/winged helix box* P3 (FOXP3), controlador chave na função e no desenvolvimento dessas células. Apresentam também alta expressão de CD25 ($CD25^{high)}$ e baixa expressão de CD127 ($CD127^{low)}$, e possuem habilidade de controlar a imunidade interferindo na geração de funções efetoras *in vivo.* Tregs são conhecidas por não serem uma população homogênea. Elas podem ser divididas em vários subgrupos que diferem funcionalmente e podem ser distinguidas pela expressão de antígenos de superfície (JONULEIT; SCHMITT, 2003; NIEDŹWIECKI et al., 2018).

Pelo menos 5 subpopulações foram identificadas até agora e são derivadas de células T *naives* sob diferentes condições (AKBAR et al., 2007; MIYARA; SAKAGUCHI, 2007; ZHANG et al., 2014):

i) Células Treg naturais (nTreg) $CD4^+$ $CD25^+$ $FOXP3^+$ surgem no timo em resposta à antígenos próprios após a interação com DCs medulares durante o processo de seleção negativa; constituem o principal subgrupo de Tregs responsáveis por manter a tolerância periférica e medeiam supressão através de mecanismos dependentes e independentes de contato célula-célula, como bloqueio da proliferação de células imunes através da ação de moléculas acessórias com atividade inibitória como proteína 4 associada à linfócito T citotóxico (CTLA-4) e gene 3 de ativação de linfócito (LAG3), fator de crescimento transformante beta (TGF-β) ligado à membrana e liberação de TGF-β e IL-10 solúveis.

ii) Tregs induzíveis (iTreg, T_h3) são células T $CD4^+$ *naives* induzidas na periferia em resposta à antígenos não-próprios, sofrem regulação de FOXP3, secretam TGF-β e IL-10 e possuem ação similar às nTregs;

iii) Células Tr1 são $CD4^+$, não expressam FOXP3 e são conhecidas por secretar IL-10 e bloquear a atividade de células T_h;

iv) Tregs $CD8^+$ $FOXP3^+$ $CD25^{high}$ produzem altos níveis de TNF-α, IFN-γ, e granzima B, no entanto bloqueiam a ativação de células T efetoras e *naives*;

v) Tregs FOXP3$^+$ produtoras de IL-17 são geradas sob estimulação do receptor de célula T (TCR) e fortemente inibem a proliferação de células T CD4$^+$ efetoras. Possivelmente, dada a alta plasticidade de células T, essas células podem se converter em T_h17 e ter sua função supressora diminuída.

Um microambiente imunossupressor na cérvice uterina é permissivo para a carcinogênese cervical induzida pelo HPV. A infiltração de células Treg em lesões precursoras e em tumores cervicais ativamente induzem anergia de células T efetoras e suprimem respostas antitumorais específicas. De fato, a contagem de células T CD4$^+$ FOXP3$^+$ TGF-β$^+$ aumenta conforme o espectro da doença também aumenta. Assim, lesões de baixo grau apresentam números baixos de Treg infiltrantes, indicando que a presença desta célula no microambiente é um fator prognóstico desfavorável (KOBAYASHI et al., 2008).

2.8 O FATOR DE TRANSCRIÇÃO *FORKHEAD BOX* P3 (FOXP3)

O fator de transcrição *forkhead box* P3 (FOXP3) pertence à família de fatores de transcrição *winged-helix*. Seu papel como amplo regulador da expressão gênica é central para a identidade e função do subgrupo mais amplamente reconhecido e bem estudado de células T imunorreguladoras, as Tregs. Estas células são definidas pela expressão constitutiva de *FOXP3*, embora essa expressão possa ser induzida transitoriamente em células não Treg sob ativação (WANG et al., 2007).

Em humanos, mutação do gene *FOXP3* leva à síndrome de imunodesregulação, poliendocrinopatia e enteropatia ligada ao X (IPEX), tipicamente fatal. Pacientes com este distúrbio genético desenvolvem inúmeras imunopatologias nos primeiros meses de vida, incluindo dermatite, enteropatia, diabetes, tireoidite e anemia (BENNETT et al., 2001). Camundongos *Scurfy*, que carregam uma mutação *nonsense* em *Foxp3* e que resulta em uma inserção de 2 pb no gene, expressam uma proteína truncada. As células Treg nestes animais são inativas, e são incapazes de restringir a hiperatividade de células T e sua produção de citocinas pró-inflamatórias (RAMSDELL; ZIEGLER, 2014). Por isso, à época de sua caracterização, a proteína Foxp3 foi chamada de scurfina (BRUNKOW et al., 2001).

O gene humano *FOXP3* está localizado no braço curto do cromossomo X (locus Xp11.23) (Figura 3). O gene inclui 11 éxons codificantes e três não-codificantes e um alto grau de conservação é visto entre o gene humano e o de camundongos, especialmente nas interfaces éxon-intron (BRUNKOW et al., 2001). A proteína FOXP3 compreende três domínios

funcionalmente importantes: domínio N-terminal com atividade reguladora e repressora transcricional, uma região contendo um dedo de zinco e um zíper de leucina importante para a homodimerização de FOXP3 e um domínio *forkhead* C-terminal, que possui atividade repressora e responsável por ligar-se ao DNA e interagir com o fator de transcrição NFAT (XIE et al., 2015).

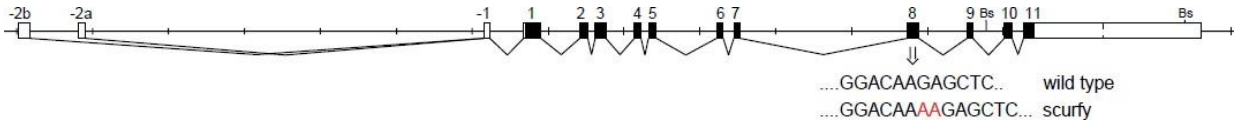

Figura 3. O gene *FOXP3.*

Caixas pretas representam éxons codificantes, enquanto éxons não codificantes são as caixas brancas. Linhas conectando os éxons representam locais onde ocorrem *splicing*. Éxon não codificantes 5' (-2a e -2b), separados por 640 pb, se unem à um terceiro éxon não codificante (-1). A mutação caracterizada pela inserção de 2 pb responsável por causar a síndrome IPEX é representada abaixo do éxon 8.

Fonte: BRUNKOW et al. (2001).

FOXP3 é capaz de se ligar a mais de 2.800 sítios genômicos, que correspondem a aproximadamente 700 a 1.119 genes envolvidos no desenvolvimento e estabelecimento de células Treg (ZHENG et al., 2007; MARSON et al., 2010). Regulando esses *loci* alvo, FOXP3 coopera funcionalmente com, ou possivelmente reforça, os padrões de expressão gênica que surgem da programação epigenética iniciada pela estimulação do receptor de células T (TCR) durante o desenvolvimento das Tregs (MORIKAWA; SAKAGUCHI, 2014).

Em humanos, os transcritos de FOXP3 sofrem *splicing* alternativo e geram múltiplas isoformas variantes: FOXP3Δ2, que não possui o éxon 2; FOXP3Δ7, que não possui o éxon 7 (e o domínio de zíper de leucina); e FOXP3Δ2'7, que não possui os éxons 2 e 7. Enquanto que a isoforma completa de FOXP3 suprime a expressão mediada por RORα, NFκB e NFAT, FOXP3Δ2 e FOXP3Δ2'7 apresentam uma inibição prejudicada destes fatores de transcrição pró-inflamatórios (RYDER et al., 2012).

Vias de sinalização mediadas pelo TCR (imperativa para a indução da transcrição), IL-2R (CD25), co-estimulação mediada por CD28, MAP quinases (MAPK) são todas importantes para o início da transcrição de *FOXP3*. Sinalização da via das SMADs induzida pelo TGF-β possui um papel crucial na expressão de *FOXP3*, especialmente na geração de iTregs. Estas cascatas recrutam vários fatores de transcrição à elementos regulatórios e ao promotor do gene *FOXP3* e possibilitam o desenvolvimento e manutenção do fenótipo Treg (LU; BARBI; PAN, 2017).

O gene *FOXP3* possui sequências não-codificantes (CNS) conservadas (CNS1, CNS2 e CNS3) com diferentes padrões de metilação. CNS1 serve como um *sensor* de TGF-β e funciona como um *enhancer*. O CNS2 é importante para estabilizar a expressão de *FOXP3*. Fatores de transcrição que compõem uma plataforma molecular conhecida como *enhanceossomo* podem se ligar as regiões conservadas e atuarem como reguladores positivos da expressão do gene (MARUYAMA et al., 2011a).

As oncoproteínas E6 e E7 do HPV induzem a ativação do promotor do TGF-β1 ligando-se à sequência de reconhecimento do fator de transcrição da proteína 1 (Sp1) (PERALTA-ZARAGOZA et al., 2006). Sabe-se que a expressão de *FOXP3* é induzida por TGF-β. Além disso, a existência do sítio de ligação de Sp1 já foi demonstrada no gene *FOXP3* (TONE et al., 2008), o que indica que o HPV pode perpetuar um estado imunossupressor através da ativação de *FOXP3*, direta ou indiretamente.

2.9 INTERLEUCINA 10

A IL-10, inicialmente denominada de fator inibitório da síntese de citocinas (CSIF), é o principal membro da superfamília IL-10, tendo sido descrita na década de 1990. A proteína humana é codificada pelo gene *IL-10* localizado no locus 1q31-1q32. Possui forma homodimérica em forma de V com um peso molecular de 37 kDa, com um comprimento total de 178 aminoácidos (aas), 160 aas de segmento maduro e 18 aas que compreendem o peptídeo sinal. Sinaliza através da ligação constante com seu receptor transmembrana tetramérico, o IL-10R. Sua expressão é regulada a nível transcricional e pós-transcricional, principalmente por fatores de transcrição ativadores da família das STATs (transdutor de sinal e ativador da transcrição) e por alterações epigenéticas como modificação de histonas (PESTKA et al., 2004; MANNINO et al., 2015).

Muitos tipos celulares podem produzir IL-10, incluindo monócitos/macrófagos, DCs, células B, células T_h2, várias subpopulações de células Treg, células T $CD4^+$ e $CD8^+$ e células NK (HEDRICH; BREAM, 2010). Células tumorais também podem produzir IL-10, como é o caso de células de carcinoma cervical (KOBAYASHI et al., 2008).

A persistência da infecção pelo HPV pode sofrer influência da secreção de IL-10. Existe uma interação entre o HPV e a IL-10, com esta citocina induzindo a expressão de proteínas precoces como E2, E6 e E7, enquanto estas proteínas virais induzem a expressão da IL-10, criando um ciclo vicioso (Figura 4) (TORRES-POVEDA et al., 2014).

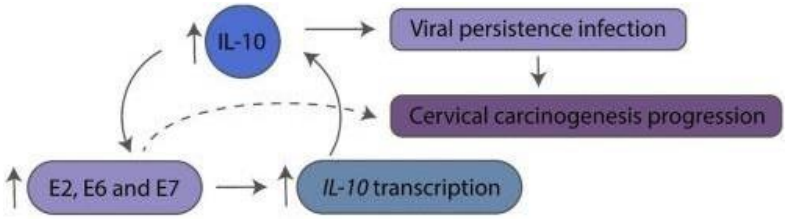

Figura 4. Consequências patológicas provocadas pela interação entre IL-10 e proteínas virais. Mecanismos interdependentes da infecção pelo HPV e produção de IL-10 pelo hospedeiro e desfecho clínico.
Fonte: BERTI et al. (2017).

Níveis cervicais elevados de IL-10 em conjunto com outros efeitos imunossupressores induzidos pelo HPV contribuem com o início do desenvolvimento de SIL, permitindo ao HPV subverter a vigilância imunológica e estabelecer persistência viral. Ao passo que SIL persiste, integração do genoma viral no genoma do hospedeiro, transformação neoplásica e imortalização ocorrem, levando à progressão tumoral (WANG et al., 2012).

Várias subpopulações de células Treg secretam IL-10, a saber: nTreg, iTreg e Treg $CD8^+$. Estas células possuem uma característica comum: a expressão de FOXP3, o que sugere que esta proteína possa ter um papel na produção de IL-10 por estas células. Por outro lado, células Treg podem não expressar este fator de transcrição, como é o caso das células Tr1, sugerindo uma produção de IL-10 de maneira FOXP3-independente (ZHANG et al., 2014). Assim, se a interação entre essas duas peças chaves da resposta imunossupressora é factível, é razoável pensar que variantes genéticas que influenciem quantitativamente ou funcionalmente FOXP3 podem também influenciar nos níveis de IL-10.

2.10 POLIMORFISMOS GENÉTICOS E *FOXP3*

SNPs são a forma mais importante e básica de variação no genoma, e está bem estabelecido que eles desempenham um papel central em doenças humanas e em resposta a patógenos infecciosos (BELL, 2002). SNPs em genes relacionados à resposta imunológica e reconhecimento de patógenos têm sido associados à susceptibilidade a doenças infecciosas (FERWERDA et al., 2007).

Um SNP não sinônimo (ou não conservativo) que ocorre em uma região codificadora pode causar uma substituição de aminoácido na proteína correspondente e modular

sua geometria ou função. SNPs não sinônimos constituem mais de 50% das variantes conhecidas por estarem envolvidas em doenças hereditárias humanas (KUMAR; HENIKOFF; NG, 2009). SNPs sinônimos ou conservativos (silenciosos) podem influenciar a expressão proteica alterando o processamento e estabilidade do mRNA e os níveis de transcrição gênica. Variação alélica e número variável de repetições em *tandem* em promotores, *enhancers* ou *silencers* podem ter um efeito significativo na transcrição, uma vez que podem alterar a estrutura dos sítios de ligação do fator de transcrição dentro de promotores e outros elementos regulatórios (HOLLEGAARD; BIDWELL, 2006).

Oda et al. (2013) revisaram o papel de diferentes SNPs em *FOXP3* em doenças de várias etiologias. De fato, SNPs ocorrem em regiões gênicas como promotora, íntrons e éxons e interferem na expressão e função de FOXP3, estando associados a desfechos clínicos desfavoráveis.

Polimorfismos de *FOXP3* estão associados à doenças infeciosas como malária e parasitemia por *P. falciparum* (KOUKOUIKILA-KOUSSOUNDA et al., 2013), tuberculose (BEIRANVAND et al., 2017), carcinoma hepatocelular relacionado à hepatite B (CHEN et al., 2012) e papilomatose respiratória recorrente severa, associada à infecção por lrHPV (KWON et al., 2017).

Dentre estes SNPs, destacamos dois deles, presentes no íntron -1 de *FOXP3*: rs3761548, caracterizado por uma substituição pontual de uma citosina por uma adenina, e rs2232365, onde há a substituição de uma adenina por uma guanina.

Associações positivas já foram descritas entre o SNP rs3761548 e psoríase (GAO et al., 2010), rinite alérgica (ZHANG et al., 2009), atopia (BOTTEMA et al., 2010), doença de Graves e tireoidite de Hashimoto (INOUE et al., 2010), LES (LIN et al., 2011), vitiligo (JAHAN et al., 2013) e aborto espontâneo recorrente (WU et al., 2012). Além disso, o SNP rs3761548 de *FOXP3* já havia sido positivamente associado à susceptibilidade em mulheres portadoras de câncer de mama triplo negativo (LOPES et al., 2014). Estes resultados contrastam com os descritos por Jiang e Ruan (2014), que não detectaram esta associação em pacientes com câncer de mama. No entanto, em uma metanálise conduzida por Cheng, Guo e Ming (2018), o SNP rs3761548 foi associado com risco para o desenvolvimento de câncer em geral quando diversas populações foram agrupadas.

O SNP rs2232365 está envolvido em condições patológicas como doença de Chron (PARK et al., 2005), psoríase (GAO et al., 2010) e aborto espontâneo recorrente (WU et al., 2012). Além disso, pacientes submetidos à transplante alogênico de células tronco hematopoiéticas e portadores deste SNP em homozigose são mais propensos a desenvolverem doença

do enxerto contra hospedeiro aguda (NAM et al., 2018). No entanto, a ocorrência de rs2232365 é fator protetor no tumor pediátrico de Wilms (OZAWA et al., 2016).

Nosso grupo de pesquisa investigou a ocorrência dos SNPs rs3761548 e rs2232365 em uma população do norte do Paraná com câncer de mama e encontrou correlações positivas entre estes SNPs e suas estruturas haplotípicas (i.e., combinações entre alelos de SNPs coherdados no mesmo cromossomo) com parâmetros prognósticos nestas pacientes (BANIN-HIRATA et al., 2017).

Entender a variabilidade imunogenética e olhar para o genoma com mais atenção são necessários não somente para compreender a notável heterogeneidade na imunovigilância antitumoral e anti-HPV, mas também para extrapolar o conhecimento gerado na bancada para a prática clínica, possibilitando o estabelecimento de novos biomarcadores e o delineamento de imunoterapias e outras modalidades de tratamento.

3 OBJETIVOS

3.1 Objetivo Geral

✓ Analisar a presença de variantes genéticas no gene do fator de transcrição FOXP3 e suas possíveis implicações na patogênese da infecção pelo HPV, das lesões precursoras do CCU e nos níveis plasmáticos e cervicais da IL-10.

3.2 Objetivos Específicos

✓ Realizar a detecção molecular e genotipagem do HPV em mulheres atendidas pelo Sistema Único de Saúde (SUS);

✓ Analisar variáveis sociodemográficas, de comportamento sexual, parâmetros ginecológicos e obstétricos em mulheres infectadas pelo HPV, portadoras de lesões precursoras do CCU e controles livres de infecção e lesões;

✓ Analisar os genótipos e haplótipos dos polimorfismos rs3761548 e rs2232365 do gene *FOXP3* em mulheres infectadas pelo HPV, portadoras de lesões precursoras do CCU e controles livres de infecção e lesões, em um estudo clínico com delineamento do tipo caso-controle;

✓ Quantificar os níveis plasmáticos e cervicais da IL-10 em mulheres infectadas pelo HPV e controles livres de infecção e correlacionar estes níveis com diferentes modelos de herança genotípica do *FOXP3*;

✓ Desenvolver um artigo de revisão narrativa da literatura que verse sobre o valor prognóstico da infiltração de células inflamatórias e do microambiente imune em tumores humanos associados à infecção pelo HPV;

4 MATERIAL E MÉTODOS

4.1 Delineamento Experimental e Aspectos Éticos

Este estudo clínico do tipo caso-controle incluiu 426 mulheres, que foram submetidas ao exame preventivo de Papanicolaou (Pap) e recrutadas por amostragem de conveniência não probabilística de vários serviços de saúde localizados em Londrina, incluindo a unidade de colposcopia ambulatorial do Consórcio Internacional de Saúde do Médio Paranapanema (Cismepar), Ambulatório de Especialidades do Hospital Universitário da Universidade Estadual de Londrina, bem como de duas Unidades Básicas de Saúde, entre março de 2015 e dezembro de 2016. Todas as participantes do estudo receberam instruções claras sobre a finalidade do estudo atual, bem como sobre os procedimentos aos quais seriam submetidas (coleta de sangue e células cervicais) antes da coleta da amostra e assinaram o termo de consentimento livre e esclarecido (TCLE). Posteriormente, foram entrevistadas em relação a vários aspectos sociodemográficos, estilo de vida sexual e histórico ginecológico e obstétrico. Pacientes que foram submetidas ao procedimento de cirurgia de alta frequência (CAF) para tratamento de SIL ou que apresentaram detecção molecular inconclusiva para HPV não foram incluídas. O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina (Londrina, PR, Brasil) (CEP/UEL 133/2012; CAAE 05505912.0.0000.5231).

4.2 Amostragem

As mulheres que foram submetidas à avaliação clínica nas consultas ambulatoriais supracitadas tiveram o epitélio cervical esfoliado com escovas citológicas. Após a coleta da amostra para citologia, as escovas citológicas foram armazenadas em 2 mL de tampão TE (10 mM Tris-HCl, 1 mM EDTA pH 8,0) a -20 °C até a análise molecular. O sangue periférico foi coletado por punção venosa em tubos de vácuo contendo ácido etilenodiaminotetraacético (EDTA) e armazenado a -20 °C até a análise. Amostras de sangue periférico total e cervicais foram obtidas de todas as 426 mulheres incluídas neste estudo. Amostras de sangue foram usadas para análise de SNPs no gene *FOXP3*, enquanto amostras cervicais foram testadas para detecção e genotipagem do HPV. O plasma separado das amostras de sangue total e o tampão TE usado para a preservação das células cervicais foram utilizados na determinação dos níveis de IL-10.

4.3 Exame Citológico das Amostras Cervicais

O esfregaço cervical foi obtido no momento do recrutamento nos serviços de saúde acima mencionados. Médicos patologistas experientes classificaram e laudaram os exames de Pap de acordo com os critérios diagnósticos do Sistema Bethesda (2001) no Laboratório do Sistema Único de Saúde (SUS). Foram consideradas neste estudo as pacientes que apresentavam LSIL, HSIL ou ausência de lesões se as amostras citológicas eram normais, ou seja, não apresentavam LSIL ou HSIL, carcinomas cervicais, células escamosas atípicas de significado indeterminado (ASCUS), ou outras atipias celulares (DA SILVA et al., 2012).

4.4 Extração do DNA Genômico

O DNA genômico foi obtido a partir de escovas citológicas cervicais usando DNAzol (Invitrogen Inc., Carlsbad, CA, EUA), de acordo com as instruções do fabricante, e armazenado a -20 °C. O DNA genômico de amostras de sangue periférico foi extraído utilizando o kit Biopur Mini Plus (Biometrix, Curitiba, PR, Brasil). A concentração de DNA foi medida por espectrofotômetro Thermo Fisher Scientific NanoDrop 2000c™ (EUA) a 260 nm e a pureza foi avaliada através da razão 260/280.

4.5 Detecção e Genotipagem do HPV

A reação em cadeia da polimerase (PCR) do HPV foi realizada utilizando os *primers* MY09 (5′-CGTCCMAARGGAWACTGATC-3’) e MY11 (5′-GCMCAGGGWCATAAYAATGG-3’), de acordo com o número de acesso do GenBank: AJ236888. Esses *primers* amplificam uma região conservada de aproximadamente 450 pb do gene *L1* do HPV (BAUER et al., 1991a). As condições de reação foram 190 nM de dNTPs, 500 nM de cada *primer*, 2 mM de $MgCl_2$, *Buffer* 1X, aproximadamente 80 ng de DNA e 1,25 U de Taq DNA polimerase (Invitrogen™, Carlsbad, CA, EUA), com uma temperatura de anelamento de 55 °C. A coamplificação do gene da β-globina humana (aproximadamente 268 pb) foi realizada como um controle de amplificação, utilizando os *primers* GH20 (5'-GAAGAGCCAAGGACAGGTAC-3′) e PC04 (5′-CAACTTCATCCACGTTCACC-3’) nas mesmas condições que a PCR de HPV (DA SILVA et al., 2012). Um controle negativo, sem DNA, foi realizado durante todos os conjuntos de reação, a fim de excluir uma possível contaminação. Além disso, um controle positivo (DNA da linhagem celular de carcinoma cervical HeLa), que contém o genoma do HPV18 integrado

ao genoma da célula, foi adotado. Além disso, para identificar o tipo de HPV, amostras positivas para HPV foram submetidas à restrição enzimática com HpyCH4V (New England Biolabs, Ipswich, MA), de acordo com a análise de amplicons da PCR do HPV através da técnica de *Restriction fragment length polymorphism* (RFLP) (SANTIAGO et al., 2006). Todos os produtos da PCR e da digestão enzimática foram analisados por eletroforese em gel de poliacrilamida não desnaturante a 10%, corado com nitrato de prata ($AgNO_3$).

4.6 GENOTIPAGEM DOS SNPS DE *FOXP3*

A PCR-RFLP foi realizada a partir do DNA genômico de sangue periférico para detectar os SNPs rs2232365 e rs3761548, de acordo com o número de acesso do GenBank NG_007392.1). No SNP rs2232365, uma adenina é substituída por uma guanina. No SNP rs3761548, uma adenina é substituída por uma citosina. Para a genotipagem de rs2232365, foram utilizados os seguintes *primers*: 5′-AGGAGAAGGAGTGGGCATTT-3’ (*forward*) e 5′-TGTGAGTGGAGGAGCTGAGG-3’ (*reverse*) (PARADOWSKA-GORYCKA et al., 2015). A genotipagem de rs3761548 foi realizada com os seguintes *primers*: 5′-GGCAGAGTTGAAA-TCCAAGC-3’ (*foward*) e 5′-CA ACGTGTGAGAAGGCAGAA-3’ (*reverse*) (HE et al., 2013). Foram utilizados PCR *Buffer* 1X (20 mM de Tris-HCl ph 8,5; 50 mM de KCl), 1 mM de $MgCl_2$, 0,1 mM de dNTP, 0,2 μM de cada *primer*, 1 U/μL de Taq DNA polimerase e 2,5 ng/μL de DNA genômico diluído em H2O ultra-pura (Milli-Q) para completar um volume final de 25 μL por tubo de reação, com adição de glicerol à 5% em volume final para a reação do SNP rs2232365. Uma amostra de controle negativo, sem DNA, foi realizada durante todos os conjuntos de reação, a fim de excluir uma possível contaminação. O protocolo de ciclagem, usado para ambos os SNPs de *FOXP3*, foi uma desnaturação a 95 °C por 5 min, 35 ciclos de 45 seg a 95 °C, 45 seg a 59 °C para o SNP g.10403A> G ou 30 seg a 65 °C para o SNP g.8048A> C, 1 min a 72 °C e 10 min de alongamento final a 72 °C. Produtos de PCR (3 μL) de rs2232365, com 249 pb, foram digeridos *overnight* a 55 °C com 1,5 unidade/reação de endonuclease de restrição *BsmBI* (New England Biolabs, Beverly, EUA), gerando dois fragmentos de 132 pb e 117 pb, correspondentes ao alelo G (Figura 5). Os produtos de PCR (6 μL) de g.8048A> C, com 155 pb, foram digeridos overnight a 37 °C com 2 unidades/reação de endonuclease de restrição *PstI* (New England Biolabs, Beverly, EUA), gerando dois fragmentos de 80 pb e 75 pb que correspondem ao alelo C (Figura 6). Todos os produtos da PCR e da digestão enzimática foram analisados por eletroforese em gel de poliacrilamida não desnaturante a 10%, corado com nitrato de prata ($AgNO_3$).

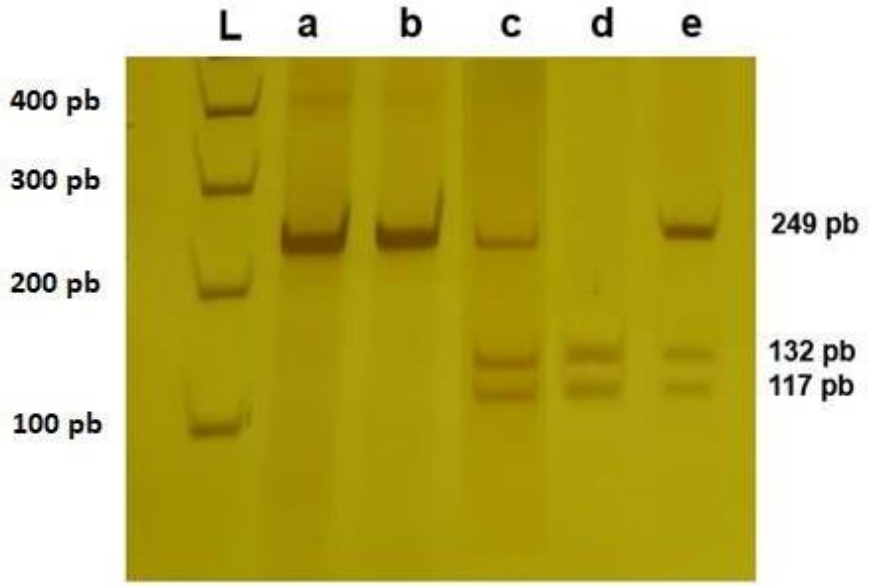

Figura 5. Perfil eletroforético do polimorfismo rs2232365.

L: Ladder 100 pb; **a**: fragmento do gene *FOXP3*; **b**: genótipo homozigoto A/A; **c**: genótipo heterozigoto A/G; **d**: genótipo homozigoto G/G; **e**: controle de clivagem.

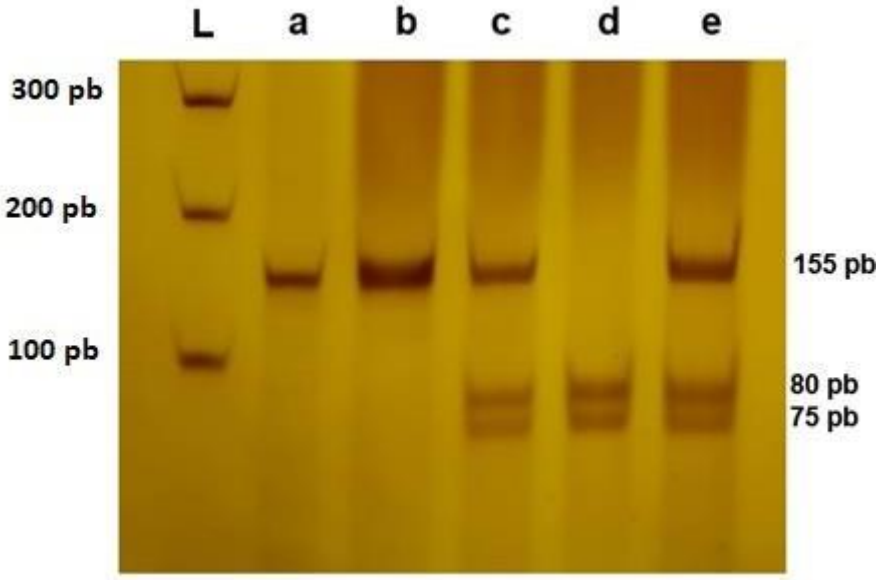

Figura 6. Perfil eletroforético do polimorfismo rs3761548.

L: Ladder 100 pb; **a**: fragmento do gene *FOXP3*; **b**: genótipo homozigoto C/C; **c**: genótipo heterozigoto C/A; **d**: genótipo homozigoto A/A; **e**: controle de clivagem.

4.7 Dosagem de IL-10

Níveis plasmáticos de IL-10 de mulheres HPV-positivas (*n* = 146) e HPV-negativas (*n* = 162), bem como níveis cervicais de IL-10 de mulheres HPV-positivas (*n* = 30) e HPV-negativas (*n* = 70) foram determinados usando o kit de ELISA para IL-10 Ready-SET-Go! (EBioscience, San Diego, CA, EUA). O *n* total desta amostragem foi de 308, considerando que as amostras cervicais das pacientes foram selecionadas a partir das amostras com diagnóstico molecular de HPV. Amostras cervicais coletadas por escovas citológicas da endo- e ectocérvice foram colocadas em tubos falcon contendo 2 mL de solução TE, que foram posteriormente centrifugadas. Os sobrenadantes foram separados e os níveis de IL-10 foram determinados usando o mesmo kit IL-10 humano. Os resultados foram expressos em picogramas por mililitro (pg/mL).

4.8 Análise Estatística

Estatística descritiva foi utilizada para caracterizar os dados da população de estudo. Análises de tabelas de contingência pelo teste de Qui-quadrado (χ^2) de Pearson foram realizadas para avaliar diferenças nas distribuições de frequência para as variáveis categóricas sóciodemográficas e clínicas e modelos de herança dos polimorfismos de *FOXP3* entre os grupos controle e caso (i.e., infecção por HPV/diagnóstico de SIL). A correção de Bonferroni foi usada como um pós-teste para controlar a taxa de ocorrência de resultados falso-positivos (erro do tipo I) decorrentes de comparações múltiplas. Frequências haplotípicas de *FOXP3* também foram comparadas com populações ao redor do mundo através do teste de $\chi 2$. Os resultados são expressos como valor absoluto e porcentagem. As variáveis contínuas comparadas em nosso delineamento (i.e., idade e níveis de IL-10) foram analisadas para desvios da distribuição gaussiana pelo teste de Kolmogorov-Smirnov e posteriormente transformadas em escala logarítmica, quando apropriado. Como a normalidade dos dados não foi assumida para a variável idade, utilizamos os testes não paramétricos de Mann-Whitney e Kruskall-Wallis com pós-teste de Dunn para identificar as diferenças de idade entre os grupos. Nesse caso, os dados são expressos como mediana e interquartis (IQR 25-75). A inferência dos haplótipos de *FOXP3* foi realizada com base nos genótipos de todas as mulheres do estudo utilizando o software PHASE versão 2.1.1 (STEPHENS; SMITH; DONNELLY, 2001; STEPHENS; SCHEET, 2005). O aplicativo *online* SNPstats (Intitut Català d'Oncologia, Barcelona, Espanha) (https://www.snpstats.net) foi utilizado para analisar o desequilíbrio de ligação (LD) entre os polimorfismos de

FOXP3 (SOLÉ et al., 2006). Análise de Variância Bidirecional (*Two-Way* ANOVA) foi realizada para verificar as interações entre a variável contínua IL-10 e variáveis categóricas (i.e., modelos de herança dos SNPs de *FOXP3* e infecção por HPV). O pós-teste de Tukey para comparações múltiplas foi usado para detectar diferenças entre os modelos. Regressão logística binária e multinomial no método *stepwise* foram realizadas para identificar entre os dados sociodemográficos e clínicos possíveis fatores confundidores que podem influenciar a análise de associação caso-controle. Regressão logística binária e multinomial controlada pelos fatores confundidores no método de entrada forçada foi empregada para predizer associações independentes entre modelos de herança dos SNPs e haplótipos (variáveis explicativas) e grupos HPV-positivas/SIL (variáveis dependentes). Correlações entre os haplótipos e diagnóstico de SIL foram avaliadas pelo coeficiente de correlação tau-b de Kendall. Todos os testes foram bicaudais, com um valor de $p<0,05$ considerado estatisticamente significativo. *Odds ratio* (OR) e intervalos de confiança de 95% (IC) foram estimados para todas as análises pertinentes. Todas as análises estatísticas foram realizadas utilizando os softwares SPSS Statistics 22.0 (SPSS Inc., Chicago, Illinois, EUA) e GraphPad Prism 7.0 para Windows (GraphPad Software, Inc., La Jolla, Califórnia, EUA).

5 PRODUÇÃO CIENTÍFICA

ARTIGO 1

Original Article

FOXP3 Intron -1 Polymorphisms are Independent Predictors of Human Papillomavirus Infection and High-Grade Squamous Intraepithelial Lesions: A Clinic-based Case-control Study

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Abstract

FOXP3 is a *bona fide* marker of the T regulatory (Treg) cell subset and drives its function and homeostasis. Its expression maintains the host immunosuppressive state that favors persistence of human papillomavirus (HPV) infection and squamous intraepithelial lesions (SIL) appearance. We evaluated the effects of the rs3761548 and rs2232365 intronic polymorphisms on HPV infection and SIL diagnosis in HPV-infected and uninfected women. HPV DNA-based detection in cervical specimens was performed by polymerase chain reaction (PCR). *FOXP3* polymorphisms were genotyped by PCR-restriction fragment length polymorphism and haplotype structures were inferred for 208 HPV-infected and 218 HPV-uninfected women diagnosed or not with low- or high-grade intraepithelial lesions. Case-control analyses were carried out by logistic regression adjusted for several socio-demographic, sexual lifestyle and clinical data. The homozygous genotype of the rs3761548 polymorphism (A/A) (related to decreased *FOXP3* expression) protected against HPV infection in women (OR_{Aj}: 0.60; 95% CI = 0.36 – 0.99; p = 0.049) this genotype is also an independent predictor of protection against HSIL development (OR_{Aj}: 0.28; 95% CI = 0.11–0.68; p = 0.006). Additionally, the homozygous genotype (G/G) of the rs2232365 polymorphism (related to increased *FOXP3* expression) was an independent risk factor for HPV infection (OR_{Aj}: 2.10; 95% CI = 1.06–4.15; p = 0.033). Haplotype analysis revealed no significant associations in our study. Our results reveal the significant and independent associations between *FOXP3* genetic variants and susceptibility to HPV infection and SIL diagnosis and their role as biomarkers of HPV infection and cervical lesions management.

Keywords: Human papillomavirus; Squamous intraepithelial lesion; *FOXP3;* Polymorphisms; Haplotypes.

Introduction

Infection by human papillomavirus (HPV) is the most common sexually transmitted infection among sexually active people. Persistent chronic infection with high oncogenic risk types (hrHPV) is a well-understood etiology of oral and anogenital cancers, particularly cervical cancer (CC), the fourth most common cancer among women (1,2). The overall global burden of HPV infection has been widely assessed by polymerase chain reaction (PCR) and hybrid capture techniques and was estimated at approximately 11-12% (3).

The hrHPVs have two main transcriptional units, E6 and E7, which encode oncoproteins essential for viral replication, allowing for tumor suppression inactivation and unchecked cell-cycle progression through p53 and retinoblastoma protein inhibition, particularly in infected keratinocytes (1). Integration of the HPV genome is an essential step in tumorigenesis and often results in the loss of regulation and overexpression of oncogenes *E6* and *E7* (4). Double-strand breaks (DSB) may enable a greater frequency of HPV-DNA integration, and are thought to be mediated by chronic inflammation-induced oxidative stress, which in turn amplify the pro-inflammatory microenvironment permissive to host DNA damage (5).

Approximately 80% of primary CC cases arise from preexisting squamous dysplasia, formally classified in the Bethesda System terminology as squamous intraepithelial lesion (SIL) and stratified as low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) (6,7). LSIL encompasses mild dysplasia/cervical intraepithelial neoplasia (CIN1), while HSIL refers to moderate and severe dysplasia (CIN 2/CIN3) and carcinoma *in situ* (7).

Persistent HPV infection does not act alone to cause SIL progression, and the immune system plays a key role. Although an effective immune response is responsible for eliminating viral infection in healthy women and inducing immunoprotection through type-1 helper T cell response activation, HPV eventually begins modulating cellular immunity towards an immune-tolerant microenvironment, characterized by $CD4^+$ $CD25^+$ $FOXP3^+$ T regulatory (Treg) cell recruitment (8).

FOXP3 is a critical regulator that maintains a heritable genetic program for $CD4^+$ $CD25^+$ Treg cells to function as suppressor T cells. Given their importance in preventing the development of autoimmunity and their therapeutic potential, the molecular mechanisms governing $CD4^+$ $CD25^+$ Treg development and function are of great interest (9).

FOXP3 is located at Xp11.23 and encodes a cluster of transcription factors that are members of the Forkhead/winged-helix family of transcriptional factors. Alternatively spliced multiple transcript variants encoding different isoforms have been identified, which may influence the differentiation or functionality of Treg cells *in vivo* (10).

Progressive up-regulation of FOXP3 has been reported in the course of SIL pathogenesis. Additionally, FOXP3 immunostaining is correlated with the expression of $p16^{INK4a}$, a key marker for the integration of high-risk HPV into host cells (11).

Genetic polymorphisms in *FOXP3* may influence protein expression and function, dampening Treg cell activity and leading to autoimmunity development (12). Intronic variants do not affect amino acid sequence but may influence protein expression through alterations in mRNA stability, levels of gene transcription, and disrupting or creating binding sites for splicing, transcriptional and other regulatory factors (13,14). *FOXP3* variants have been positively associated with several disorders such as endometriosis, idiopathic arthritis, atopy, Crohn's disease, unexplained recurrent spontaneous abortion, diabetes, and breast cancer [*as reviewed in* (12)].

Moreover, *FOXP3* loci were associated with some infectious and parasitic diseases, including tuberculosis, malaria and chronic hepatitis B (15–17). To date, no studies have investigated *FOXP3* polymorphisms in HPV infection and SIL development.

Therefore, the present study was conducted to evaluate the involvement of the *FOXP3* intron -1 single nucleotide polymorphisms (SNPs) rs2232365 and rs3761548 in HPV infection and SIL pathogenesis in a South Brazilian cohort, known to present a complex pattern of genetic and ethnic admixture.

Material and Methods

Study design and ethical approval

This clinic-based case-control study included 426 women, who underwent outpatient cytology testing and were recruited by non-probability convenience sampling from several health services in Londrina (Paraná, Southern Brazil), including the ambulatory colposcopy facility of the International Consortium of Health of the Middle Paranapanema (Cismepar), University Hospital and Clinic Center of the State University of Londrina, and two Basic Health-care Units, between March 2015 and December 2016. All study subjects received clear instructions regarding the purpose of the current study and the procedures which they would be subjected (cervical and blood collection) prior to sample collection and signed formal consent. Next, each

subject was interviewed for several socio-demographic, sexual lifestyle and gynecological and obstetric background factors. Patients who underwent the loop electrosurgical excision procedure for SIL treatment or presented an inconclusive molecular detection for HPV were excluded. The present study was approved by the Institutional Ethics Committee Involving Humans of the State University of Londrina (Londrina, PR, Brazil) (CEP/UEL 133/2012; CAAE 05505912.0.0000.5231).

Sampling

Cervical epithelial scrapings were obtained from women undergoing clinical evaluation in outpatient appointments. After sample collection for cytology, the cytobrushes were stored in 2 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) at -20 °C until molecular analysis. Peripheral blood was collected by venipuncture into vacutainer tubes containing EDTA and stored at -20^{o} C until analysis. Cervical and peripheral blood samples were obtained from women included in this study. Blood samples were used for *FOXP3* SNP analysis, while cervical samples were tested for HPV detection and genotyping.

Cytological examination of cervical samples

Cervical smears were obtained at the time of enrollment. Experienced pathologists graded and reported *Pap* smears according to the Bethesda System (2001) diagnosis criteria at the Public Health System Laboratory. Patients were deemed to have LSIL, HSIL or negative for intraepithelial lesion and malignancy (NILM) if cytology samples presented normal morphology. Patients without cervical abnormalities were not indicated as having low- or high-grade squamous intraepithelial lesions, cervical carcinomas, atypical squamous cells of undetermined significance (ASCUS), or other atypical squamous cells (18).

Genomic DNA extraction

Genomic DNA was obtained from cervical cytobrushes using DNAzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and stored at -20^{o} C. Genomic DNA from peripheral blood samples was extracted using the Biopur Mini Plus Kit (Biometrix, Curi-

tiba, PR, Brazil). The DNA concentration was measured with a NanoDrop 2000c™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 260 nm and purity was assessed by measuring the 260/280 ratio.

HPV detection and genotyping

HPV detection was carried out using the MY09 (5′-CGTCCMAARGGAWACTGATC-3′) and MY11 (5′-GCMCAGGGWCATAAYAATGG-3′) primers, according to the GenBank Accession number: AJ236888. These primers were designed to amplify a conserved region of approximately 450 base pairs (bp) of *L1* in HPV (19). Reaction conditions were 190 nM of dNTPs, 500 nM of each primer, 2 mM of $MgCl_2$, 1× of buffer (20 mM of Tris-HCl PH 8,5; 50 mM of KCl), approximately 80 ng of DNA and 1.25 U of Taq polymerase (Invitrogen), with an annealing temperature of 55 °C. Co-amplification of the human β-globin gene (approximately 268 bp) was performed as an amplification control, using primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PC04 (5′-CAACTTCATCCACGTTCACC-3′) (18), under the same conditions as used in HPV PCR. A negative control sample, with no DNA, was included in all reaction sets to exclude possible contamination. A positive control consisting of DNA from the HeLa cervical cancer cell lineage, which contains the HPV18 genome integrated into cell genome, was used. Additionally, to identify the HPV type, HPV-positive samples were subjected to an enzymatic restriction with HpyCH4V (New England Biolabs, Ipswich, MA, USA) (20). PCR and digested products were analyzed by 10% non-denaturing polyacrylamide gel electrophoresis, stained with silver nitrate.

FOXP3 genetic polymorphisms genotyping

PCR restriction fragment length polymorphism analysis was carried out using peripheral blood genomic DNA to detect rs2232365 and rs3761548 SNPs, according to GenBank Accession number NG_007392.1. In rs2232365 SNP, an adenine is replaced by a guanine. In rs3761548 SNP, a cytosine is replaced by an adenine. For rs2232365 genotyping, the following primers were used: 5′-AGGAGAAGGAGTGGGCATTT–3′ (forward) and 5′-TGTGAGTGGAGGAGCTGAGG–3′ (reverse) (21). The rs3761548 genotyping was performed with the following primers: 5′-GGCAGAGTTGAAATCCAAGC–3′ (forward) and 5′-CA ACGTGTGAGAAGGCAGAA–3′ (reverse) (22). Briefly, the reaction contained 1X of PCR buffer (20mM of Tris-HCl PH 8.5; 50mM of KCl), 1 mM of $MgCl_2$, 0.1mM of dNTP, 0.2μM of each primer,

1 U/μL of Taq DNA polymerase, and 2.5 ng/μL of genomic DNA diluted in ultra-pure H2O (Milli-Q) to a final volume of 25 μL per reaction tube, with 5% glycerol added to the rs2232365 reaction. A negative control sample, with no DNA, was included in all reaction sets to exclude possible contamination. The cycling protocol, used for both *FOXP3* SNPs, was a denaturation at 95°C for 5 min, 35 cycles of 45 sec at 95°C, 45 sec at 59°C to rs2232365 or 30 sec at 65°C to rs3761548, 1 min at 72°C, and 10 min of final elongation at 72°C. PCR products (3 μL) of rs2232365, which were 249 bp, were digested overnight at 55°C with 1.5 U/reaction of the *BsmBI* restriction endonuclease (New England Biolabs, Beverly, USA), which generated two fragments of 132 and 117 bp corresponding to allele G. The PCR product (6 μL) of rs3761548, which was 155 bp, were digested overnight at 37°C with 2.0 U/reaction of the *PstI* restriction endonuclease (New England Biolabs), generating two fragments of 80 and 75 bp that correspond to allele C. All PCR and digested products were analyzed by 10% non-denaturing polyacrylamide gel electrophoresis and silver nitrate staining.

Statistical analysis

Descriptive statistics were used to characterize the study population data. Analyses of contingency tables by Pearson's Chi-square (χ^2) test were used to evaluate differences in the frequency distributions of selected socio-demographic and clinical categorical variables and *FOXP3* polymorphisms inheritance models between controls and case groups (i.e., HPV status/SIL diagnosis). Bonferroni correction was used as a post-hoc test to address the issue of false positive (type I error) findings arising from multiple comparisons. *FOXP3* haplotype frequencies were also compared with those of populations worldwide using χ^2 test. The results are expressed as absolute values and percentage. The continuous variable distribution compared in our design (age) was tested for Gaussian distribution by the Kolmogorov-Smirnov test and normalized in logarithmic scale; when normality was not observed, we used the non-parametric Mann-Whitney test and Kruskal-Wallis test with Dunn's post-hoc test to identify age differences between groups. In this case, data are expressed as the median and interquartile range (IQR 25-75). Inference of recombination sites between *FOXP3* alleles of women studied was performed using PHASE software version 2.1.1 (23,24). The web-based application SNPstats (Institut Català d'Oncologia, Barcelona, Spain) (https://www.snpstats.net) was used to analyze linkage disequilibrium (LD) between *FOXP3* polymorphisms (25). Binary and multinomial logistic regression with a stepwise method were performed to identify among socio-demographic

and clinical data as possible confounding factors that may have biased the case-control association analysis. Binary and multinomial logistic regression controlled by confounders in the forced entry method was employed to predict independent associations between SNP inheritance models and haplotypes as explanatory variables and case groups (i.e., HPV/SIL) as dependent variables. Correlations between haplotypes and SIL diagnosis were evaluated by Kendall's tau-b rank correlation coefficient. All tests were two-tailed, with a p value<0.05 considered statistically significant. Odds ratio (OR) and 95 % confidence intervals (CI) were estimated. Statistical analyses were carried out using SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL , USA) and GraphPad Prism 7.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Sample characterization according to HPV infection, socio-demographic and clinical data

First, 426 women were included in the study and categorized as HPV-infected (208/48.8%) and HPV-uninfected or controls (218/51.2%). The median age of HPV-infected patients was 34 years (IQR 26-46), while that of HPV-uninfected women was 42 (IQR 32-51), showing a significant difference ($p < 0.001$). The socio-demographic, sexual lifestyle, gynecological, and obstetric data of these patients are summarized in Tables 1 and 2.

When HPV-infected women and HPV-uninfected women were compared, a higher frequency of HPV was observed in women younger than 24 years old ($p < 0.001$), receiving <1 minimum wage ($p = 0.022$), single ($p = 0.001$), smokers ($p = 0.017$), and nulliparous ($p = 0.034$). Moreover, HPV infection was more common among women with an age at first intercourse of less than 17 years ($p = 0.018$) and had at least 4 sexual partners during their lifetime ($p < 0.001$).

For convenience, HPV-infected women were divided into two groups, according to the carcinogenic potential of the HPV type screened: hrHPV infection and lrHPV infection. We found no association between socio-demographic, sexual lifestyle, gynecological, and obstetric data and low/high-risk infection (data not shown).

Additionally, women in this study were divided into three groups based on cytological abnormalities detected and classified according to the Bethesda System classification as follows: NILM (control group) (304/74%), LSIL (30/7.3%) and HSIL (77/18.7%) groups. The median age of controls subjects was 40 years (IQR 30-51), LSIL was 34 years (IQR 22-42), and HSIL was 34 years (IQR 28-46). HSIL showed a significant difference from the controls ($p = 0.033$).

Socio-demographic, sexual lifestyle, gynecological, and obstetric data of these patients are presented in Tables 3 and 4. A positive significant association was found between LSIL and HSIL diagnosis and age lower than 24 years ($p = 0.023$), LSIL and hormonal contraceptive method use ($p = 0.009$), and LSIL and abortion ($p = 0.011$). HSIL diagnosis was associated with women receiving <1 minimum wage ($p < 0.001$), smoking status ($p = 0.006$), at least 4 sexual partners during their lifetime ($p = 0.007$) and sexual partners within the past 6 months ($p = 0.010$).

FOXP3 polymorphisms and HPV infection/SIL susceptibility

The minor allele frequency (MAF) of rs3761548 was consistent with that reported by the 1000 Genome Project (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). The MAF of rs2232365 differed from the global frequency found by the 1000 Genome Project. However, when analyzing different populations separately, the MAF of rs2232365 corresponded to Ad Mixed American and East Asian populations.

Case-control analysis were performed to assess the influence of *FOXP3* intronic polymorphisms on HPV infection and SIL susceptibility. Genotype distributions and *p* values for the $\chi 2$ test are shown in Table 5. Associations were tested considering codominant model (heterozygotes or variant homozygotes versus wild-type homozygotes), dominant model (heterozygotes and variant homozygotes versus wild-type homozygotes), recessive model (variant homozygotes versus wild-type homozygotes and heterozygotes) and overdominant model (variant heterozygotes versus variant and wild-type homozygotes).

The rs3761548 codominant, recessive and overdominant models were associated with an increased risk of HPV infection. In agreement with these results, when the models were adjusted by binary logistic regression, only the recessive model (i.e., assessing the A/A genotype effect) was independently associated with virus infection, displaying a protective role ($OR_{Adj} = 0.60$; 95% CI = 0.36–0.99; $p = 0.049$) (Table 6). rs2232365 did not reach significance in the $\chi 2$ test, while multivariate analyses detected an association of codominant model with an increased risk of HPV infection ($OR_{Adj} = 2.10$; 95% CI = 1.06–4.15; $p = 0.033$). Association studies between low/high-risk HPV infection and *FOXP3* polymorphisms showed no significant association (data not shown).

Furthermore, we investigated whether *FOXP3* polymorphisms are involved in LSIL and HSIL pathogenesis. The rs3761548 codominant and recessive models showed a protective role in SIL development. Indeed, A/A genotype carriers were more likely to present with normal cytology. This finding was confirmed by multinomial logistic regression, with the A/A genotype found

to be an independent protective factor in HSIL development (OR_{Adj} = 0.28; 95% CI = 0.11–0.68; p = 0.006). The rs3761548 overdominant model (i.e., assessing the C/A genotype effect) was found to be an independent risk factor for HSIL development (OR_{Adj} = 1.77; 95% CI = 1.01–3.11; p = 0.046) (Table 6).

FOXP3 haplotype structures and HPV infection/SIL susceptibility

Four possible haplotype combinations were investigated in our experimental design and their distribution are shown in Table 7.

Analysis of linkage disequilibrium among rs2232365 and rs3761548 showed these SNPs are not good surrogate markers of each other (D' = 0.16; r^2 = 0.15). Once they are inherited in the same chromosome with a high independency level, it is important to assess their combined effects.

In the association study of *FOXP3* haplotypes, the following models were analyzed: AC dominant (AA, GC, and GA carriers versus AC carriers), AC recessive (AA, GC, and GA carriers versus ACAC), AA dominant (AC, GC, and GA carriers versus AA carriers), AA recessive (AC, GC, and GA carriers versus AAAA carriers) GC dominant (AC, AA, and GA carriers versus GC carriers), GC recessive (AC, AA, and GA carriers versus GCGC), GA dominant (AC, AA, and GC carriers versus GA carriers), and GA recessive (AC, AA, and GC carriers versus GAGA).

The predominant haplotype was AC in our patient cohort, while the less frequent haplotype was the AA. The haplotype frequencies of controls were compared to those of African, European, American, and Asian populations, using publicly available data from the 1000 Genome Project obtained through the web-based application LDlink (26). *FOXP3* haplotype frequencies differed significantly (p <0.0001 by $\chi 2$ test) from those of the overall population. As expected, frequencies from our cohort matched with Utah Residents with Northern and Western European Ancestry (p = 0.826) and British in England and Scotland (p = 0.154) populations.

In the present study, no significant association between haplotypes and HPV infection or degrees of SIL diagnosis was detected. At this point, for statistical purposes, we grouped our SIL samples into two groups: NILM and SIL diagnosis. We found a trend of association between the AA haplotype in the dominant model (p = 0.077) and strong trend between the AA haplotype in the recessive model (p = 0.058) with SIL protection. Additionally, correlation analysis revealed a strong trend for a negative correlation between the AA haplotype in dominant model and SIL diagnosis (r = - 0.94; p = 0.056).

Discussion

We analyzed the *FOXP3* rs3761548 and rs2232365 genetic polymorphisms in outpatients infected with HPV, carrying CC premalignant lesions and HPV negative and lesion-free controls. The main findings of our study were that the A/A homozygous genotype of the rs3761548 polymorphism is a good predictor of protection from HPV infection in women; this genotype is also an independent protection predictor for HSIL development. Furthermore, women carrying the G/G homozygous genotype of the rs2232365 polymorphism are more prone to be infected with the virus (Supplementary Fig. 1).

Our results show for the first time the rs3761548 and rs2232365 SNPs as biomarkers of HPV infection and SIL diagnosis. This is the first genetic association study investigating the influence of *FOXP3* polymorphisms in such pathological conditions, despite more than one decade of consistent evidence that $FOXP3^+$ cells infiltration plays a marked deleterious role in the course of HPV-associated carcinogenesis (27,28).

We found a positive association between the variables age lower than 24 years, low monthly income, single marital status, smoking status, nulliparity, early age at first sexual intercourse and high number of sexual partners during the lifetime with HPV infection. Although nulliparity was associated with infection in this study, this result may be biased by the younger age of patients. SIL diagnosis was associated with age lower than 24 years, low monthly income, smoking status, hormonal contraceptive method, abortion, early age at first sexual intercourse and high number of sexual partners.

Because infection by oncogenic HPV is a necessary but not sufficient cause of CC (29), the variables described above are all well-known risk factors associated with HPV infection and HPV-related cervical oncogenesis, and our findings confirm the accumulated epidemiological data reported by our research group and extensive medical literature (30–34). Social factors may not be directly involved in lesions and cancer pathology, but may predispose individuals to HPV infection and the transition to cervical malignancy because of restricted access to educational, prevention and sexual and reproductive health tools (35).

FOXP3 is an X-linked gene that encodes a transcription factor, the most specific and reliable biomarker of Treg cells. Indeed, canonical *FOXP3* expression is essential for driving $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cell function (36,37). Previous studies demonstrated that mutations in *FOXP3* may cause immune response impairment and contribute to autoimmune diseases development (38), infectious processes (15), and cancer (39).

Evidence from psoriatic patients showed that $CD4^+$ $CD25^+$ $FOXP3^+$ T cells carrying the rs3761548 A/A genotype have lower FOXP3 levels caused by slowed transcription/expression and reduced luciferase reporter activity when allele A was present (12). C>A substitution causes binding loss to E47 and c-Myb transcription factors, impairing *FOXP3* gene transcription.

We found a significant association between allele C of rs3761548 as confirmed in the codominant, recessive and overdominant models and an increased risk of HPV infection. In agreement with this, the A/A genotype was found to have independent protective effects against infection. The A/A genotype was more frequent among NILM patients and was found to be independently associated with protection against HSIL development. The C/A genotype was associated with an increased risk of HSIL development. A recent meta-analysis found that the rs3761548 polymorphism, particularly the recessive model (A/A vs C/A + C/C: OR = 1.45, 95%CI = 1.03–2.02, $p = 0.03$), is associated with an increased cancer risk in the overall population (40).

We inferred that the C allele increases HPV susceptibility by up-regulating *FOXP3* expression, but the opposite may be true: the A allele may protect against HPV infection and high-grade dysplasia by decreasing *FOXP3* expression, inducing down-regulation of Treg-mediated immunoregulatory mechanisms and establishing effective anti-viral and anti-tumor cellular immunity. This assumption is in agreement with a study showing high frequencies of HPV 16-specific Treg cells were strongly correlated with HPV persistent infection and HSIL progression (41).

The roles of *FOXP3* in neoplastic cells are still conflicting. FOXP3 expression has been detected in cervical cancer cell lines and is correlated with $p16^{INK4a}$ expression, suggesting a protumor action (42). Moreover, inhibited *FOXP3* expression in Siha HPV-16 positive cervical cancer cells following lentivirus mediated RNA interference showed inhibited cell proliferation and invasiveness, apoptosis induction and cell cycle arrest (42). Conversely, reports have indicated that *FOXP3* acts as a tumor suppressor gene and is a favorable prognostic factor in several tumors, including HPV-positive tonsillar squamous cell carcinomas (43,44).

Bioinformatic analysis revealed that rs2232365 is located in a putative binding site for the transcription factor GATA-3 (45). Additionally, this transcription factor binds the promoter region of *FOXP3* only when the allele A is present. Binding of GATA-3 inhibits *FOXP3* expression, and this transcription factor must be removed from the promoter for *FOXP3* expression to occur (46). Next, G/G carriers lose their GATA-3 binding site, enabling *FOXP3* gene transcription. Accordingly, women infected with *Mycobacterium tuberculosis* carrying the G/G genotype of rs2232365 SNP show 2.28 folds higher *FOXP3* expression than A/A carriers (15). In the present study, the G/G genotype of the rs2232365 polymorphism was an independent predictor of HPV

infection. An, A>G substitution may contribute to Treg FOXP3^{+} generation and exert a detrimental role in the anti-HPV immune response. *FOXP3* plays a dual role in cell proliferation, with the G/G genotype showing a protective role in severe recurrent respiratory papillomatosis (i.e., lrHPV infection-related) (47).

In our cohort, the *FOXP3* haplotype distribution of the control group differed significantly from that of the global population reported in the 1000 Genomes Project data, which did not verify these values in the Brazilian population. Our results confirm those of Banin-Hirata et al. (2017) findings, who found a significant difference between *FOXP3* haplotypes frequencies of neoplasia-free patients among a Southern Brazilian population and different countries and continents. Examining the populations separately, the *FOXP3* haplotype distribution matched that of European populations. Accordingly, Southern Brazil region display the highest proportion of European ancestry among geopolitical regions of the country (49).

No previous studies have investigated the effects of the rs3761548 and rs2232365 haplotypes on HPV and SIL susceptibility. This is the first study to report a lack of association between *FOXP3* haplotype and HPV infection or SIL diagnosis.

Finally, the present study revealed that the A/A genotype of the rs3761548 SNP is a good predictor of prognosis in CC premalignant lesions. Additionally, the G/G genotype of the rs2232365 SNP is an independent risk factor for HPV infection. Further studies of the associations between immune gene variations and infection and SIL susceptibility may reveal the molecular immunopathogenesis of CC and lead to the establishment of new health prevention practices based on molecular screening.

Disclosure

The authors declare that there are no conflicts of interest to disclose.

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Table 1 Socio-demographic data of HPV-uninfected and HPV-infected women.

Variable	HPV-uninfected		HPV-infected		*p* value*
	n = 218[a]	(%)	*n* = 208[a]	(%)	
Ethnicity					0.223
Caucasian	115	(52.8)	94	(45.2)	
Non-Caucasian	97	(44.5)	101	(48.6)	
Missing data	6	(2.7)	13	(6.2)	
Age range (years)					**<0.001**
≤ 24	12	(5.5)	44[b]	(21.2)	
25 – 34	53	(24.3)	63	(30.3)	
35 – 44	54	(24.8)	44	(21.2)	
45 – 54	60	(27.6)	28	(13.4)	
≥ 55	36	(16.5)	27	(13.0)	
Missing data	3	(1.3)	2	(0.9)	
Educational level[c]					0.836
Incomplete elementary	64	(29.4)	62	(29.8)	
Complete elementary	26	(11.9)	24	(11.5)	
Incomplete secondary	29	(13.3)	29	(13.9)	
Complete secondary	69	(31.7)	66	(31.7)	
Incomplete higher education	7	(3.2)	6	(2.9)	
Complete higher education	17	(7.8)	9	(4.3)	
Missing data	6	(2.7)	12	(5.9)	
Monthly income					**0.022**
<1 minimum wage	55	(25.2)	73[b]	(35.1)	
1–< 3 minimum wages	140	(64.2)	101	(48.6)	
> 3 minimum wages	17	(7.8)	15	(7.2)	
Missing data	6	(2.8)	19	(9.1)	
Marital status					**<0.001**
Single	22	(10.1)	51[b]	(24.6)	
Married/civil partner	157	(72.0)	119	(57.2)	
Divorced	27	(12.4)	24	(11.5)	
Widowed	11	(5.0)	14	(6.7)	
Missing data	1	(0.5)	0	(0.0)	
Smoking status					**0.017**
No	180	(82.6)	150	(72.1)	
Yes	35	(16.0)	52[b]	(25.0)	
Missing data	3	(1.4)	6	(2.9)	
Knowledge about HPV					0.502
No	42	(19.3)	48	(23.0)	
Have ever heard	117	(53.7)	100	(48.1)	
Yes	55	(25.2)	49	(23.6)	
Missing data	4	(1.8)	11	(5.3)	

*****Analysis by two-sided Pearson's Chi-square (χ^2) test, with $p < 0.05$ considered significant.

[a] For association analysis between HPV-uninfected and HPV-infected women not all 426 patients were included, with variations depending on the characteristic analyzed.

[b] $p < 0.05$, tested by Bonferroni post-hoc test for multiple comparisons.

[c] Based on Brazilian educational system.

[d] Based on Brazilian minimum wage (approximately US$ 250.00).

Table 2 Sexual lifestyle, gynecological and obstetric data of HPV-uninfected and HPV-infected women.

Variable	HPV-uninfected		HPV-infected		*p* value*
	n = 218[a]	(%)	*n* = 208[a]	(%)	
Contraceptive method					0.433
No	120	(55.0)	96	(46.2)	
Yes, hormonal	66	(30.3)	77	(37.0)	
Yes, condom	24	(11.0)	27	(13.0)	
Yes, both	8	(3.7)	8	(3.8)	
Missing data	0	-	0	-	
Parity					**0.034**
0	19	(8.7)	35[b]	(16.8)	
1	37	(17.0)	45	(21.6)	
2	71	(32.6)	47	(22.6)	
3	45	(20.6)	47	(22.6)	
4	24	(11.0)	18	(8.7)	
> 5	22	(10.1)	16	(7.7)	
Missing data					
Abortion					0.973
No	155	(71.1)	135	(64.9)	
Yes	44	(20.2)	38	(18.3)	
Missing data	19	(8.7)	35	(16.8)	
Age at first sexual intercourse (years)					**0.018**
≤ 17 years	108	(49.5)	125[b]	(60.1)	
≥ 18 years	109	(50.0)	79	(38.0)	
Missing data	1	(0.5)	4	(1.9)	
Age at menarche (years)					0.694
≤ 11	47	(21.6)	51	(24.5)	
12	51	(23.4)	54	(26.0)	
13	53	(24.3)	45	(21.6)	
≥ 14	65	(29.8)	55	(26.5)	
Missing data	2	(0.9)	3	(1.4)	
Sexual partners during lifetime					**<0.001**
1	87	(39.9)	45	(21.6)	
2-3	69	(31.7)	68	(32.7)	
≥ 4	58	(26.6)	82[b]	(39.4)	
Missing data	4	(1.8)	13	(6.3)	
Sexual partners within the past 6 months					0.098
0	30	(13.8)	28	(13.5)	
1	176	(80.7)	148	(71.2)	
≥ 2	2	(0.9)	8	(3.8)	
Missing data	10	(4.6)	24	(11.5)	

***** Analysis by two-sided Pearson's Chi-square (χ^2) test, with $p < 0.05$ considered significant.

[a] For association analysis between HPV-uninfected and HPV-infected women not all 426 patients were included, with variations depending on the characteristic analyzed.

[b] $p < 0.05$, tested by Bonferroni post-hoc test for multiple comparisons.

Table 3 Socio-demographic data of SIL-negative and SIL-positive women.

Variable	NILM		LSIL		HSIL		*p* value*
	n = 304[a]	(%)	*n* = 30[a]	(%)	*n* = 77[a]	(%)	
Ethnicity							0.180
Caucasian	159	(52.3)	10	(33.3)	30	(39.0)	
Non-Caucasian	139	(45.7)	15	(50.0)	39	(50.6)	
Missing data	6	(2.0)	5	(16.7)	8	(10.4)	
Age range (years)							**0.023**
≤ 24	29	(9.5)	8[b]	(26.7)	15[b]	(19.5)	
25 – 34	82	(27.0)	6	(20.0)	25	(32.5)	
35 – 44	72	(23.7)	7	(23.3)	16	(20.8)	
45 – 54	72	(23.7)	3	(10.0)	12	(15.6)	
≥ 55	48	(15.8)	2	(6.7)	9	(11.6)	
Missing data	1	(0.3)	4	(13.3)	0	-	
Educational level[c]							0.299
Incomplete elementary	85	(28.0)	9	(30.0)	28	(36.4)	
Complete elementary	38	(12.5)	1	(3.3)	8	(10.4)	
Incomplete secondary	41	(13.5)	6	(20.0)	11	(14.3)	
Complete secondary	104	(34.2)	8	(26.7)	17	(22.1)	
Incomplete higher education	10	(3.3)	0	-	3	(3.9)	
Complete higher education	21	(6.9)	2	(6.7)	1	(1.3)	
Missing data	5	(1.6)	4	(13.3)	9	(11.6)	
Monthly income[d]							**<0.001**
< 1 minimum wage	83	(27.3)	6	(20.0)	38[b]	(49.4)	
1 – < 3 minimum wages	191	(62.8)	16	(53.3)	25	(32.5)	
> 3 minimum wages	24	(7.9)	2	(6.7)	2	(2.6)	
Missing data	6	(2.0)	6	(20.0)	12	(15.5)	
Marital status							0.246
Single	45	(14.8)	8	(26.7)	18	(23.4)	
Married/civil partner	205	(67.4)	14	(46.7)	46	(59.7)	
Divorced	38	(12.5)	4	(13.3)	8	(10.4)	
Widowed	16	(5.3)	3	(10.0)	5	(6.5)	
Missing data	0	-	1	(3.3)	0	-	
Smoking status							**0.006**
No	248	(81.6)	18	(60.0)	50	(64.9)	
Yes	53	(17.4)	9	(30.0)	24[b]	(31.2)	
Missing data	3	(1.0)	3	(10.0)	3	(3.9)	
Knowledge about HPV							0.271
No	62	(20.4)	4	(13.3)	20	(26.0)	
Have ever heard	167	(54.9)	13	(43.4)	30	(39.0)	
Yes	72	(23.7)	9	(30.0)	19	(24.7)	
Missing data	3	(1.0)	4	(13.3)	8	(10.3)	

NILM: negative for intraepithelial lesion and malignancy; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

*****Analysis by two-sided Pearson's Chi-square (χ^2) test, with $p < 0.05$ considered significant.

[a] For association analysis between NILM and SIL-positive women not all 411 patients were included, with variations depending on the characteristic analyzed.

[b] $p < 0.05$, tested by Bonferroni post-hoc test for multiple comparisons.

[c] Based on Brazilian educational system.

[d] Based on Brazilian minimum wage (approximately US$ 250.00).

Table 4 Sexual lifestyle, gynecological and obstetric data of SIL-negative and SIL-positive women.

Variable	NILM		LSIL		HSIL		*p* value*
	n = 304[a]	(%)	*n* = 30[a]	(%)	*n* = 77[a]	(%)	
Contraceptive method							**0.009**
No	156	(51.3)	2	(6.7)	24	(31.1)	
Yes, hormonal	94	(31.0)	17[b]	(56.7)	29	(37.7)	
Yes, condom	34	(11.1)	5	(16.6)	9	(11.7)	
Yes, both	10	(3.3)	3	(10.0)	3	(3.9)	
Missing data	10	(3.3)	3	(10.0)	12	(15.6)	
Parity							0.367
0	37	(12.1)	3	(10.0)	10	(13.0)	
1	58	(19.1)	2	(6.7)	19	(24.7)	
2	90	(29.6)	10	(33.3)	14	(18.2)	
3	66	(21.7)	8	(26.7)	17	(22.1)	
4	26	(8.6)	5	(16.6)	11	(14.3)	
> 5	27	(8.9)	2	(6.7)	6	(7.7)	
Missing data	0	-	0	-	0	-	**0.011**
Abortion							
No	214	(70.4)	15	(50.0)	54	(70.1)	
Yes	53	(17.4)	12[b]	(40.0)	13	(16.9)	
Missing data	37	(12.2)	3	(10.0)	10	(13.0)	
Age at first sexual intercourse (years)[c]							**0.021**
≤ 17 years	159	(52.3)	20	(66.7)	50	(64.9)	
≥ 18 years	144	(47.4)	8	(26.7)	25	(32.5)	
Missing data	1	(0.3)	2	(6.6)	2	(2.6)	
Age at menarche (years)							0.370
≤ 11	65	(21.4)	8	(26.7)	22	(28.6)	
12	80	(26.3)	5	(16.7)	20	(26.0)	
13	67	(22.0)	10	(33.3)	18	(23.4)	
≥ 14	90	(29.6)	6	(20.0)	16	(20.8)	
Missing data	2	(0.7)	1	(3.3)	1	(1.2)	
Sexual partners during lifetime							**0.007**
1	111	(36.5)	6	(20.0)	11	(14.3)	
2-3	92	(30.3)	11	(36.7)	23	(29.9)	
≥ 4	97	(31.9)	9	(30.0)	34[b]	(44.2)	
Missing data	4	(1.3)	4	(13.3)	9	(11.6)	
Sexual partners within the past 6 months[c]							**0.010**
0	42	(13.9)	0	-	14	(18.2)	
1	249	(81.9)	19	(63.3)	43	(55.8)	
≥ 2	5	(1.6)	2	(6.7)	3	(3.9)	
Missing data	8	(2.6)	9	(30.0)	17	(22.1)	

NILM: negative for intraepithelial lesion and malignancy; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

*****Analysis by two-sided Pearson's Chi-square (χ^2) test, with $p < 0.05$ considered significant.

[a] For association analysis between NILM and SIL-positive women not all 411 patients were included, with variations depending on the characteristic analyzed.

[b] $p < 0.05$, tested by Bonferroni post-hoc test for multiple comparisons.

[c] This variable was not used in multiple comparisons because its column proportion is equal to zero or one.

Table 5 *FOXP3* genotype distribution considering HPV infection status/SIL diagnosis and inheritance models testing.

FOXP3 Genotypes	HPV-uninfected		HPV-infected		*p*-value*	NILM		LSIL		HSIL		*p* value*
	n = 218[a]	(%)	*n* = 208[a]	**(%)**		*n* = 304[a]	(%)	*n* = 30[a]	(%)	*n* = 77[a]	(%)	
rs3761548												
Codominant model					**0.018**							**0.036**
C/C	63	(31.7)	66	(32.2)		90	(31.0)	11	(36.7)	27	(35.5)	
C/A	81	(40.7)	105[b]	(51.2)		126	(43.4)	12	(40.0)	42	(55.3)	
A/A	55	(27.6)	34	(16.6)		74[b]	(25.5)	7	(23.3)	7	(9.2)	
Dominant model					0.908							0.552
CC	63	(31.7)	66	(32.2)		90	(31.0)	11	(39.3)	27	(35.5)	
C/A + A/A	136	(68.3)	139	(67.8)		200	(69.0)	17	(60.7)	49	(64.5)	
Recessive model					**0.007**							**0.008**
AA	55	(27.6)	34	(16.6)		74[b]	(25.5)	5	(17.9)	7	(9.2)	
C/C + C/A	144	(72.4)	171[b]	(83.4)		216	(74.5)	23	(82.1)	69	(90.8)	
Overdominant model					**0.034**							0.175
C/C + A/A	118	(59.3)	100	(48.8)		164	(56.6)	16	(57.1)	34	(44.7)	
C/A	81	(40.7)	105[a]	(51.2)		126	(43.4)	12	(42.9)	42	(55.3)	
rs2232365												
Codominant model					0.426							0.582
A/A	46	(22.0)	34	(17.3)		56	(19.2)	6	(21.4)	14	(19.4)	
A/G	129	(61.7)	124	(63.3)		188	(64.6)	15	(53.6)	42	(58.3)	
G/G	34	(16.3)	38	(19.4)		47	(16.2)	7	(25.0)	16	(22.2)	

Table 5 *FOXP3* genotype distribution considering HPV infection status/SIL diagnosis and inheritance models testing (continued).

Dominant model					0.239							0.962
A/A	46	(22.0)	34	(17.3)		56	(19.2)	6	(21.4)	14	(19.4)	
A/G + G/G	163	(78.0)	162	(82.7)		235	(80.8)	22	(78.6)	58	(80.6)	
Recessive model					0.412							0.289
GG	34	(16.3)	38	(19.4)		47	(16.2)	7	(25.0)	16	(22.2)	
A/A + A/G	175	(83.7)	158	(80.6)		244	(83.8)	21	(75.0)	56	(77.8)	
Overdominant model					0.749							0.361
A/A + G/G	80	(38.3)	72	(36.7)		103	(35.4)	13	(46.4)	30	(41.7)	
A/G	129	(61.7)	124	(63.3)		188	(64.6)	15	(53.6)	42	(58.3)	

NILM: negative for intraepithelial lesion and malignancy; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.

*****Analysis by two-sided Pearson's Chi-square (χ^2) test, with $p < 0.05$ considered significant.

[a] For association analysis not all 426 patients were included because of technical issues in *FOXP3* genotyping.

[b] $p < 0.05$, tested by Bonferroni post-hoc test for multiple comparisons.

Table 6 Case-control multivariate analysis considering HPV infection status/SIL diagnosis and inheritance models.

Models	Case groups [OR (CI 95%)]		
	HPV infection[a]	LSIL[b]	HSIL[b]
rs3761548			
Codominant model			
C/C	Reference	Reference	Reference
C/A	1.21 (0.75-1.95)	1.11 (0.42-2.93)	1.22 (0.66-2.27)
A/A	0.67 (0.38-1.20)	0.68 (0.19-2.40	**0.31*** (0.12-0.83)
Dominant model			
CC	Reference	Reference	Reference
C/A + A/A	1.00 (0.64-1.56)	0.94 (0.38-2.32)	0.87 (0.48-1.55)
Recessive model			
C/C + C/A	Reference	Reference	Reference
A/A	**0.60*** (0.36-0.99)	0.64 (0.20-2.00)	**0.28*** (0.11-0.68)
Overdominant model			
C/C + A/A	Reference	Reference	Reference
C/A	1.42 (0.94-2.15)	1.30 (0.54-3.11)	**1.77*** (1.01-3.11)
rs2232365			
Codominant model			
A/A	Reference	Reference	Reference
A/G	1.43 (0.83-2.45)	1.11 (0.35-3.50)	0.98 (0.46-2.10)
G/G	**2.10*** (1.06-4.15)	1.85 (0.50-6.88)	1.55 (0.63-3.77)
Dominant model			
A/A	Reference	Reference	Reference
A/G + G/G	1.55 (0.91-2.63)	1.29 (0.43-3.84)	1.11 (0.53-2.30)
Recessive model			
A/A + A/G	Reference	Reference	Reference
G/G	1.59 (0.92-2.73)	1.71 (0.62-4.72)	1.57 (0.79-3.09)
Overdominant model			
A/A + G/G	Reference	Reference	Reference
A/G	1.00 (0.65-1.53)	0.81 (0.33-1.98)	0.78 (0.43-1.39)

*$p<0.05$

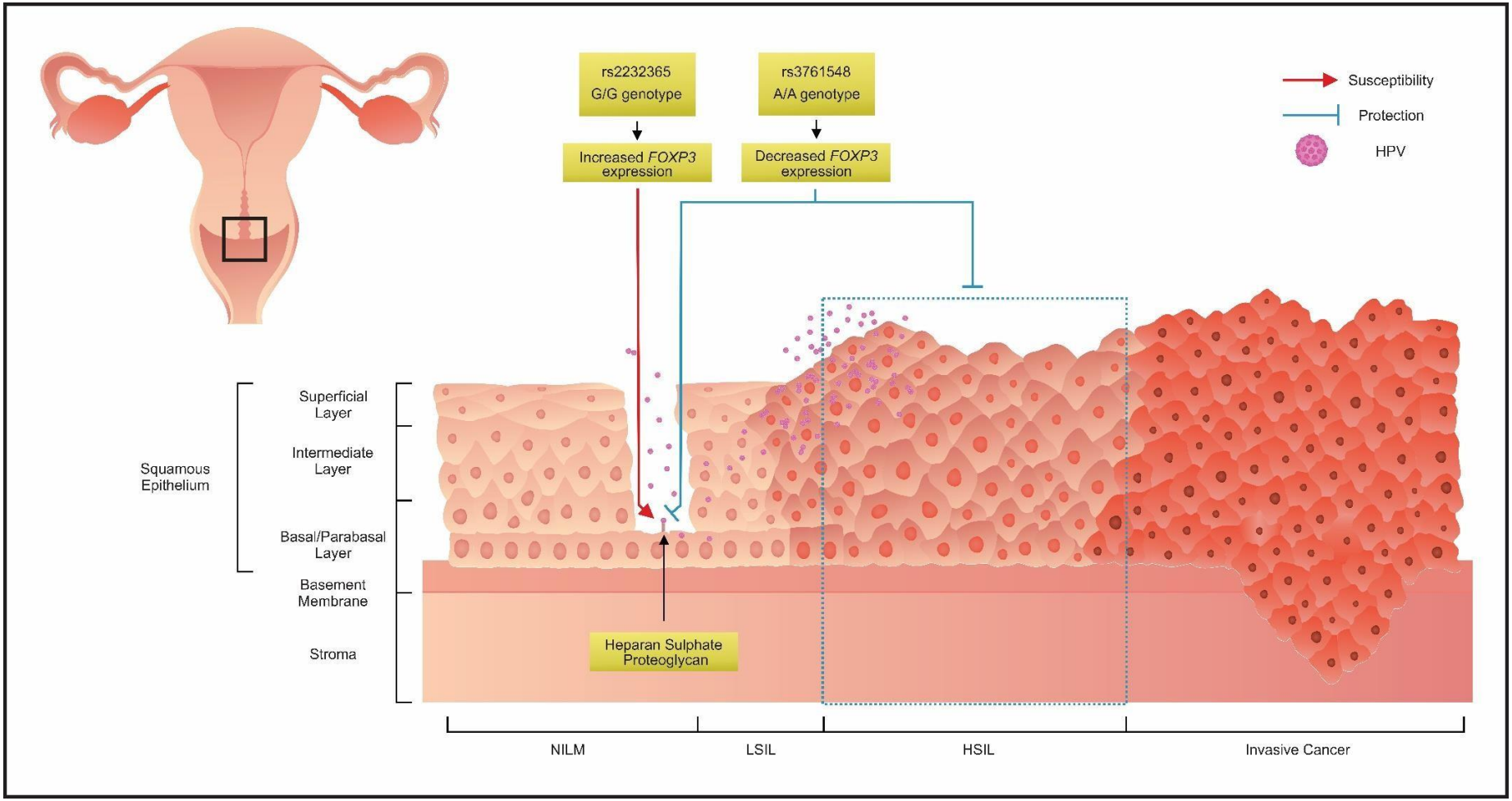
[a] OR (odds ratio) and CI (confidence interval) 95% estimated by binary logistic regression with "HPV-uninfected group" as reference and controlling by age and partners during lifetime.

[b] OR (odds ratio) and CI (confidence interval) 95% estimated by multinomial logistic regression with "NILM group" as reference and controlling by age and abortion.

Table 7 Haplotype distribution considering HPV infection status/SIL diagnosis.

Haplotypes	Haplotype count [*n* (%)]				
	HPV-uninfected	HPV-infected	NILM	LSIL	HSIL
A/A	52 (23.9)	37 (17.8)	69 (22.7)	5 (16.7)	10 (13.0)
A/C	143 (65.6)	144 (69.2)	200 (65.8)	18 (60.0)	58 (75.3)
G/A	120 (55.0)	125 (60.1)	176 (57.9)	19 (63.3)	42 (54.5)
G/C	64 (29.4)	69 (33.2)	91 (29.9)	9 (30.0)	29 (37.7)

NILM: negative for intraepithelial lesion and malignancy; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion.



Supplementary Figure 1. Roles of *FOXP3* variants related to *FOXP3* expression in HPV infection and cervical carcinogenesis. A theoretical model of *FOXP3* genetic variants actions has been postulated. *FOXP3* displays a deleterious role in HPV-associated carcinogenesis, facilitating HPV infection and cervical lesions progression through immune response negative regulation. *FOXP3* genetic variants associated to *FOXP3* mRNA expression may act in a dual manner during HPV infection and cervical lesions pathogenesis.

ARTIGO 2

Short Communication

FOXP3 Intronic Variants do not Influence Interleukin-10 Levels in HPV-infected Women

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Abstract

This study investigated the possible influence of *FOXP3* intron -1 polymorphisms in HPV plasmatic and cervical IL-10 levels of women positive to HPV-DNA presence. Our findings show that there are no significant differences between *FOXP3* genotypes and IL-10 levels. We suggest that the presence of *FOXP3* polymorphic alleles does not affect IL-10 expression. This is the first report indicating this lack of relation, and the interference of *FOXP3* genetic variants in others FOXP3^{+} Treg cells products should be sought.

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Short Communication

Forkhead box P3 (FOXP3) transcription factor is the master gene that regulates $CD4^+$ $CD25^+$ T cell regulatory (Treg) development and function, and frame-shit mutations in this gene were already reported to cause several diseases associated with an overreactive immune response (1). This T cell subset is known to display an immunosuppressive phenotype, characterized by high production of immunoregulatory cytokines, mainly TGF-β and IL-10 (2). These cytokines induce an immune tolerant microenvironment in viral infections, inhibiting viral clearance through immune response negative regulation (3); IL-10 plays a role even more harmful, inducing HPV-immune escape (4). Single nucleotide polymorphisms (SNPs) in *FOXP3* regulatory regions may alter Treg activity by regulating *FOXP3* gene transcription (5). Thus, we hypothesized *FOXP3* SNPs rs3761548 and rs2232365 in intron -1 region might influence on IL-10 plasmatic and cervical levels in an HPV infection-context. This case-control study included 308 women who underwent outpatient cytology testing and were recruited by non-probability convenience sampling from several health services located in Londrina (Paraná, Southern Brazil), including the ambulatory colposcopy facility of the International Consortium of Health of the Middle Paranapanema (Cismepar), University Hospital and Clinic Center of the State University of Londrina, as well as from two Basic Health-care Units, between March 2015 and December 2016. All study subjects received clear instructions about the purpose of the current study, as well as about the procedures they were going to be submitted to (cervical and blood collection) prior to sample collection, and signed a formal consent. The research protocol was approved by the Institutional Ethics Committee Involving Humans of the State University of Londrina (Londrina, PR, Brazil) (CEP/UEL 133/2012; CAAE 05505912.0.0000.5231). Cervical and peripheral blood samples were obtained from all women. Blood and cervical samples were used for *FOXP3* SNPs analysis and HPV detection, respectively, according to Cezar-dos-Santos et al. procedures (unpublished data). Sample number (*n*) for IL-10 plasmatic levels measurement was 146 HPV-infected and 162 uninfected women. From these, IL-10 cervical levels of HPV infected (n =30) and uninfected (n = 70) were also determined using the Human IL-10 ELISA Ready-SET-Go!™ (eBioscience, San Diego, CA, USA). Cervical samples collected by cytobrushes from endo- and ectocervices were placed into falcon tubes containing 2 mL TE solution, which were subsequently centrifuged. The supernatants were separated and IL-10 protein levels were determined using the same Human IL-10 kit. The results were expressed as picograms per milliliter (pg/mL). Regarding data analysis, the Kolmogorov-Smirnov normality test was performed to analyze deviations from

Gaussian distribution. Two-Way Analysis of Variance (ANOVA) was carried out to verify interactions between the continuous variable IL-10 and *FOXP3* inheritance models, considering HPV infection. Tukey's post-hoc test for multiple comparisons was used to detect differences between models. All tests were two-tailed with a significance level set at 0.05. Analyses were carried out using the GraphPad Prism 7.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). The *FOXP3* genotype distribution and HPV-infection case-control association analysis for researched SNPs were described by us previously (unpublished data). Considering the within-subjects analysis of *FOXP3* genotypes and inheritance models, we were not able to detect any significant differences both in plasmatic and cervical IL-10 levels of HPV-infected and uninfected women by ELISA method (Table 1). It is important to highlight that we found a significant difference in between-subjects analysis, that is, IL-10 cervical levels between HPV-infected versus uninfected women by Two-Way ANOVA followed by Bonferroni's correction, irrespective of *FOXP3* genotypes (data not shown). This predictable finding is related to HPV infection, reported earlier by our research group (6). Maynard et al. (7) showed a dual reporter mouse system of the genes encoding *IL-10* and *Foxp3* to track Treg subsets based on coordinate or differential expression of these genes. They found that a subset of $Foxp3^-$ -expressing Treg cells might secrete IL-10. It leads us to suggests that although *IL-10* is a putative binding site to FOXP3 transcription factor (8), IL-10 transcription and translation may occur in a FOXP3-independent manner, which would explain why the *FOXP3* SNPs (known to alter gene expression) do not interfere with IL-10 production. Therefore, further studies are required to better clarify whether FOXP3 transcription factor and *IL-10* gene present a physical interaction and may somehow influence on IL-10 production in different disease contexts.

Disclosure

The authors declare that there are no conflicts of interest to disclose.

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Table 1 IL-10 plasmatic and cervical levels in HPV-infected and uninfected women stratified by inheritance models.

FOXP3 Models	IL-10 Plasmatic Levels mean ± SD*		IL-10 Cervical Levels mean ± SD*	
	HPV-uninfected (*n* = 162)	HPV-infected (*n* = 146)	HPV-uninfected (*n* = 30)	HPV-infected (*n* = 70)
rs3761548				
Codominant				
C/C	3.53 ± 2.15	3.47 ± 1.69	3.57 ± 2.25	5.31 ± 3.42
C/A	3.74 ± 2.21	3.49 ± 1.91	3.93 ± 2.41	6.02 ± 2.90
A/A	3.76 ± 2.36	3.58 ± 1.86	3.90 ± 2.72	5.84 ± 3.23
Dominant				
C/A+A/A	3.75 ± 2.25	3.51 ± 1.89	3.92 ± 2.52	5.95 ± 2.98
Recessive				
C/C+C/A	3.65 ± 2.18	3.48 ± 1.82	3.80 ± 2.33	5.83 ± 2.99
Overdominant				
C/C+A/A	3.62 ± 2.22	3.50 ± 1.73	3.76 ± 2.50	5.64 ± 3.22
rs2232365				
Codominant				
A/A	3.77 ± 2.48	3.97 ± 1.71	3.67 ± 1.84	4.67 ± 2.96
A/G	3.70 ± 2.12	3.61 ± 1.89	4.01 ± 2.79	5.70 ± 3.01
G/G	3.31 ± 1.95	2.87 ± 1.52	4.56 ± 3.40	6.40 ± 3.45
Dominant				
A/G+G/G	3.60 ± 2.08	3.42 ± 1.83	4.13 ± 2.90	5.81 ± 3.04
Recessive				
A/A+A/G	3.72 ± 2.22	3.69 ± 1.85	3.91 ± 2.54	5.53 ± 2.98
Overdominant				
A/A+G/G	3.55 ± 2.24	3.36 ± 1.68	4.03 ± 2.55	5.53 ± 3.16

Comparisons of the IL-10 levels between models were performed by Two-Way ANOVA with Tukey's post-hoc test.

All analysis presented $p>0.05$.

The variation of the *n* among groups is due to samples availability (i.e., adequate blood samples, TE availability).

ARTIGO 3

Review

Immune Signature in Human Papillomavirus-associated Cancers: a Useful Tool for Prognostic Assessment

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Abstract

The human papillomavirus (HPV) is an oncogenic virus responsible for the squamous cells' malignant transformation. Cervical cancer (CC) is the most incident HPV-related neoplasia in women worldwide, but other types of cancer associated with HPV persistent infection such as vagina, vulva, penis, anus and head and neck have alarmed public health authorities. Search for prognosis and prediction markers have been the focus of researchers during the past years. Demonstration that infiltrating T cells have a great impact on patient clinical outcome changed the tumor immunology area. Indeed, analysis of immune cells *in situ* has been shown to be superior to traditional staging systems in cancer prediction, and the pattern of host immune response may be critical for predict survival rates and patient therapy. In addition, it has been proved that systemic levels of immune cells also contribute to disease outcome. High mortality rates due to failures in an accurate tumor staging demand that immune markers must be used as a prognostic tool in clinical practice, especially in CC cases. In this review we will discuss the general aspects of HPV infection and prevalence and how the density of cells from innate and adaptive immune response might influence the progression and the prognosis of HPV-related cancers.

Background

Immune system plays a crucial role in eliminating the progression and formation of incipient neoplasia, late-stage tumors and micrometastases (1). The concept of immunological surveillance was hypothesized by Frank MacFarlane Burnet and Thomas Lewis more than five decades ago and proposes the body is constantly monitored by an ever-alert immune system, which is responsible for detecting and destroying neoplastic cells and nascent tumors (2,3). Immunocompetent hosts are capable of eradicating highly immunogenic tumors, a process referred as "cancer immunoediting''. This is a complex concept that integrates two properties of tumor immune response: host-protective and tumor-promoting effects (4).

The fact that immunodeficiency is highly associated with a higher risk of cancer demonstrates the role of cancer immune surveillance (4). In addition, the great majority of cancers related to a weakened immune response are virus-induced, indicating the efforts of the immune system in virus-infected cells elimination and a decrease of viral burden are required for tumor control (1).

Human Papillomavirus (HPV) are tumor viruses that undergo their vegetative reproductive cycle within, and in synchrony with, squamous epithelial cells. These viruses are able to create a replication competent environment that allows viral genome amplification and unregulated cell proliferation, which eventually lead to cancer promotion and progression in individuals who cannot resolve high-risk HPV (hrHPV) infection (5).

Cell immortalization reflects the HPV capability to inactivates the most known canonical pathways mediated by the tumor-suppressor proteins p53 and pRb (retinoblastoma) protein through *E6* and *E7* viral oncogenes unregulated transcription, respectively. It results in DNA damage control inhibition, apoptosis evasion and forced entry into S phase (6).

The evaluation of cancer progression is performed longitudinally through the Tumor-Node-Metastasis (TNM) classification, which is useful in estimate patients prognosis in a variety of solid cancers (7). Still, prognostic potential of this traditional classification in predicting the cancer outcome and response therapy is limited, once tumors with the same histological stage may exhibit different behaviors (8).

This deficiency in precisely predicting the cancer outcome is due to the automated conduct of evaluating only tumor cells behavior and ignoring the fact that the tumor progression is strongly associated with immune cells infiltration (9). Location, density and functional orientation of

different immune cell populations (termed the ‘immune contexture*’)* in a wide variety of human cancers, including cervical cancer (CC), have important implications for identification of useful biomarkers in predicting prognosis and response to chemotherapy and radiotherapy (10).

Indeed, studies have shown the potential of the tumor-infiltrating lymphocytes (TIL) presence in determining patient survival in CC, corroborating the fact that a strong interaction between the immune system and tumor exists and drives the prognosis (11,12).

Therefore, this review outlines the main innate and adaptive immune cells that infiltrate HPV-associated tumors and their clinical utility and significance of these cells as predictive and prognostic factors.

General features of HPV-related neoplasia

The IARC Monograph 100B stated the following cancer sites as having strong evidence for a causal relationship with HPV: uterine cervix, penis, vulva, vagina, anus and oropharynx, including base of the tongue and tonsils (13).

CC is undoubtedly the most incident gynecologic malignancy afflicting women worldwide (14), which makes it the most comprehensively studied cancer related to chronic HPV infection. The uterine cervix is lined by stratified squamous epithelium, which comprises the exocervix and mucus-secreting columnar epithelium, known to coat the endocervical canal. The transition between these tissues is termed the squamocolumnar junction, an area thought to be at great risk of viral neoplastic transformation (15).

The natural history of HPV-induced CC (i.e., the course of disease or infection from onset to resolution) covers reversible alterations suspected on cytological examination of exfoliated cervical cells (*Pap* smear test) and confirmed on histopathological analysis of cervical tissue. These changes range from no neoplastic changes detected in the squamous epithelium, to distinct states of cellular abnormalities that may lead to CC (16).

Milder or more uncertain changes have been labeled ASCUS (atypical squamous cells of undetermined significance) (17). Pre-cancer lesions are named cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesion (SIL), according to Richart and Bethesda systems, respectively. In Richart system, CIN 1 corresponds to mild dysplasia and CIN 2 to moderate dysplasia. CIN 3 encompasses both severe dysplasia and carcinoma *in situ* (CIS) (18).

Bethesda Nomenclature System for Cervicovaginal Cytology grouped this terminology in LSIL

(i.e., CIN 1) and HSIL (i.e., CIN 2/CIN 3/CIS), in order to communicate clinically relevant information to physicians (19,20). LSIL is characterized by productive viral infection and presence of koilocytes in the suprabasal cell layers, following several clinical courses. Most of LSIL (60%) are transient, showing a spontaneous regression within 12-24 months. 30% of LSIL persist, and about 10% progress to HSIL within 2-years follow-up (21). About 41–67% of HSIL, 16–32% of LSIL and 6–27% of ASCUS are estimated to be HPV 16/18-positive, the more prevalent HPV genotypes (22).

When HSIL is not properly treated, the development of ICC may take years to decades. However, this transition may occur in <1 year in about 10% of patients. Squamous cervical carcinomas (SCC) arise from ectocervix and are more frequent ones, accounting for approximately 75% of ICC cases. Otherwise, adenocarcinomas (ADC) (about 20-25% of CC) are more likely to arise from endocervix (15). Adenosquamous, small cell or neuroendocrine, serous papillary, and clear cell carcinomas of the cervix present non-HPV-associated histologies and are less common histological subtypes (15).

The most common HPV types in ICC are, in order of decreasing prevalence, HPV 16, 18, 45, 31, 33, 58, 52, 35, 59, 56, 6, 51, 68, 39, 82, 73, 66 and 70. In general, HPV 16 are the predominant type found in SCC (46–63%) followed by HPV 18 (10–14%), 45 (2–8%), 31 (2–7%) and 33 (3–5%). In ADC, the prevalence is significantly lower (76.4%) than in SCC (87.3%), and HPV 18 is the predominant type (37–41%), followed by 16 (26–36%) and 45 (5–7%) (14).

Besides the persistent hrHPV infection, other recognized risk factors for CC are early age of sexual activity, multiple sexual partners, other sexually transmitted diseases (e.g., *Chlamydia trachomatis* infection) cigarette smoking, oral contraceptive use, human immunodeficiency virus infection and impaired immunological status (23).

HPV prevalence in other anogenital cancers is heterogeneous: 90% in vulvar intraepithelial neoplasia (VIN) and basaloid or warty cancers, being the HPV 16 the most prominent type found in vulvar cancer; 85% of vaginal cancer, with HPV 16 being detected in 60% of invasive tumors. HPV is detected in basaloid and warty cancers of the penis, but rarely in SCC (HPV 16 is predominant in invasive penile cancers); 80-96% in anal cancer, in which HPV 16 again is the major type detected (5).

Oropharyngeal cancers (OPC) are also strongly related to HPV infection, in contrast to other head and neck squamous cell carcinomas (HNSCC). In fact, HPV fulfills the criteria for causality of this particular cancer, reaching 72% of detection in OPC (24). HPV 16 is the most common type found, but other HPV types such as 18, 31, 33, and 35 may also be detected (5).

The TNM classification for staging CC is recommended by UICC and includes the International Federation of Gynecology and Obstetrics (FIGO) and AJCC (American Joint Committee on Cancer) systems. According to these systems, the most important factors for CC risk prediction are tumor size, depth of cervical stromal invasion, parametrial invasion, histologic type and lymph node/distant metastasis (7,25).

The standard treatment for FIGO stage IIB CC (i.e., locally advanced stage) preconized by The National Comprehensive Cancer Network (NCCN) guidelines is cisplatin-based chemoradiotherapy (26). Radical hysterectomy with pelvic lymph node dissection followed by adjuvant radiotherapy or radical radiotherapy alone was proven to have similar rates of recurrence, progression-free survival, overall survival (OS) and local control rates (LCR) in FIGO stage IIB CC patients, although radical radiotherapy is a safer tool associated with fewer treatment-related complications (27). An alternative for these patients that have shown promising results is neoadjuvant chemotherapy followed by a radical hysterectomy, which improves the long-term disease-free survival (DFS) and OS of patients with locally advanced CC stage IIB (28).

The aforementioned prognostic factors of CC are the most important ones to prognosis assessment. In addition, identification of tissue biomarkers has been proven instrumental in predicting the response to treatment and survival rates. $p16^{INK4}$ (cyclin-dependent kinase inhibitor 2A/multiple tumor suppressor 1) is a routine biomarker associated with CC progression (29). Ki-67, a proliferation antigen used to assess the proliferation index in several cancers, is useful to prognosis prediction in HPV-related cancers, especially when combined with p16 expression (29–31). Additionally, TIL is a biomarker that present relevance in CC and is associated with clinical outcomes (32).

Inflammatory cells and immune microenvironment in HPV-associated tumors

The link between inflammation and cancer was made for the first time by the physician Rudolf Virchow in 1863, who observed leucocytes infiltration in neoplastic tissues and hypothesized that “lymphoreticular infiltrate” reflected the origin of cancer at sites of chronic inflammation (33). Our current knowledge has corroborated this hypothesis, and accumulated evidence clearly show that an inflammatory microenvironment is a crucial component for all tumors, playing a decisive role in tumorigenesis process, including initiation, promotion, malignant conversion, invasion, and metastasis (34).

Infiltration of tumor-associated immune cells is a powerful tool and a clinically relevant prognostic biomarker for oncologists. The proof lies in the fact that an absent or slight inflammatory reaction in cervical sections in CC in stages IB and IIA is an independent risk factor for recurrence (35).

Neutrophils. Neutrophils immunostaining in cervical tissue is increased in patients with CIN 2/3 and restricted to the stroma rather than normal cervix (36). Intratumoral (ITM) (i.e., infiltrating the tumor nests) staining for myeloperoxidase (MPO) (i.e., a marker for neutrophils presence) is showed in tumor tissues from CC patients (37). Presence of ITM and peritumoral (PTM) (i.e., in the stroma at the migrating border of tumor nests) tumor-associated neutrophils (TANs) $CD66b^+$ is an independent poor prognostic factor for recurrence-free survival (RFS) in patients with early-stage CC (38).

TANs infiltration is also reported in laryngeal squamous cell carcinoma microenvironment. High ITM TAN infiltration is associated with high CXCL5 expression (i.e., known to support TANs migration) and advanced clinical stages in those patients (39). TANs infiltration is related to poor clinical outcomes in tongue squamous cell carcinoma (TSCC), and high infiltration of TANs $CD66b^+$ is observed in the ITM region and in the invasive front of oral squamous cell carcinomas (OSCC), although there is no correlation with clinical features (40,41). In that way, a recent study showed that neutrophils infiltration is a risk enhancing factor for HPV+ HNSCC patients (42).

Systemic inflammation may be determinant in cancer progression, and an elevated peripheral neutrophil-to-lymphocyte ratio (NLR) has been associated with worse OS and event-free survival (EFS) in CC (43). Association with OS was observed in early, locally advanced and advanced disease, and patients receiving chemoradiotherapy. Neutrophilia also is related to worse OS and PFS in HNSCC locally advanced patients after cisplatin-based chemoradiotherapy (44) and worse OS in penile cancer (45).

Interestingly, the presence of neutrophils-like MDSC (myeloid-derived suppressor cells) in a small set of CC patients expressing granulocyte colony-stimulating factor (G-CSF) (which have a poor clinical outcome) is related with chemotherapy resistance (46). MDSCs are polymorphonuclear and monocytic immature myeloid cells known to be potent negative regulators of anti-tumor immune response (especially T and natural killer cells activity and dendritic cells maturation) and stimulate angiogenesis and vascular remodeling (47,48).

Individuals with cutaneous squamous cell carcinoma, also related to HPV infection (49), show

high circulating granulocytic MDSC (G-MDSC) frequencies associated with 2B/3 tumors. Tumors ≥5 mm thick have increased number of total and peritumorally localized $CD66b^+$ leucocytes (i.e., which comprises neutrophil, G-MDSC or eosinophil populations, although eosinophils were not observed in histopathological analyses) (50).

Furthermore, IL-17-expressing neutrophils promote tumor growth and are associated with poor prognosis in early stage SCC (51). A great staining for neutrophils $IL\text{-}17^+$ in stromal sections of SCC and HNSCC patients was also reported (51). A high number of neutrophils showed a trend toward poor survival in early-stage disease and were associated with the absence of vaso-invasion when TNM parameters were analyzed.

Although neutrophils have a classical role in a protective immune response, large evidence has shown TANs acting paracrinally to support local tumor growth, progressive disease and distant metastasis (52).

Eosinophils. A dense ITM and stromal infiltrate of local eosinophilia was found in CC patients and associated with better prognosis, although they were present in PTM region as well (53). Improved survival due to stromal infiltrate of eosinophils was also described by Kapp and LiVolsi (1983).

In agreement with this observation, higher stromal tissue eosinophilia is associated with 5-year survival rate for stage IB CC women (55). Conversely, stromal eosinophilia was associated with invasion in women with HSIL. Once is hard to distinguish between HSIL and microinvasive or invasive SCC in small or superficial biopsy specimens, the stromal eosinophilia may be an adjunctive criterion for distinction of HSIL and ICC (56).

Moreover, worse OS is observed in CC with high eosinophilic infiltration (57). Eosinophils presence in oral cancers also have an unfavorable prognosis, however, in penile cancers, their presence represents improved survival (58,59).

Mast cells. Tryptase-positive ($MC_{t)}$ and tryptase-chymase ($MC_{tc)}$ positive mast cells (MC) are present in the normal uterine cervix and tumor tissues as well, widely interspersed within the stroma and close to blood vessels (60).

MC infiltration is high in CIN 3 and ICC than in normal sections; abundant ITM MC_t infiltration is seen in ICC, as well as in almost all grades of dysplasia, while MC_{tc} is limited at the PTM region, being not found within the tumors (60). Inflammatory mediators as the extracellular

matrix degrading-serine proteases tryptase and chymase are known to be potential metastatic and angiogenic factors produced by mast cells (61).

Wilk et al. (2009), investigating the MC density in normal cervix, CIN, and ICC samples, found that microvessel density (MVD) increased from normal cervix samples through LSIL and HSIL to ICC, with a significant increase of chymase-positive MC in ICC than in normal samples. Considering the whole study population, correlations were found between MVD and MC density.

Interestingly, MC show the same behavior in oral cancers. Intralesional and perilesional regions of OSCCs contain increased MVD and MC infiltration than normal mucosa (63). Furthermore, these two parameters are positively correlated. MC quantification of tumor-free mucosa and squamous cell carcinoma of the oral cavity, the pharynx, and larynx showed MC density was significantly higher in tumor-associated stroma than that observed in the tumor-free stroma (64).

Alaeddini et al. (2017) were not able to detect an association between angiogenesis/lymphangiogenesis, MC density and the histologic risk assessment model (i.e., which includes the lymphocytic infiltration) in high-grade and low-grade/intermediate-grade OSCCs.

In general lines, MC density is associated with favorable effects for oral tumors angiogenesis, mainly by neovascularization of the subepithelial lamina propria, probably by secretion of angiogenic factors as VEGF and matrix metalloproteinases (e.g., MMP-9) (66).

Langerhans cells. Presence of Langerhans cells (LC) infiltration (i.e., a subpopulation of the epithelial dendritic cell family) in women carrying koilocytotic atypia, CIN, microinvasive SCC and invasive SCC have been described, being seen mostly in ITM region, and in a smaller number situated outside of the tumor nests (67). LC density is significantly increased as the grade of CIN advanced. SCC and ADC patients containing ITM and PTM LC infiltration treated by radiotherapy present better 5-year survival rates than patients without LC infiltration (68).

In fact, LC infiltration seems to participate in radiation curability. Stage III SCC and ADC patients demonstrate better 10-year survival rates than controls. Once LC presence is correlated to T cell infiltration in tumor tissues, possibly LC improve local response to radiotherapy trough T cell-induced anti-tumor response (69). Accordingly, $CD11c^{+}$ $CD11b^{-}$ dendritic cells densities increase in first week of chemoradiation treatment in stage IBI-IIIB CC (70).

Presence of LC among tumor cells and into the connective tissue around tumor also predicts a significantly better 5-year survival rate in stage III ADC patients treated with radiotherapy alone (71). Density of immature LC $CD1a^+/LAG^+$ is decreased in transformation zone (TZ) in comparison with exocervix of SIL patients; increased density of inefficient LC is correlated to HSIL development (72). It is reasonable considering the role of LC in immune surveillance of the squamous epithelium and the majority of SIL occur at metaplastic TZ.

Similarly, there is a decreased number of LC $CD1a^+$ in VIN (73). HIV-positive women presenting SIL have a decrease of LC density, which may predispose SIL development (74). High degree of LC infiltration in larynx cancer is associated with less local and regional recurrence, less cervical lymph node metastasis, longer DFS, and less clinical N-positivity (i.e., tumor cells that has spread to the lymph nodes) (75).

In HSCC, ITM LC number is greater in oral cavity than in the oropharynx, hypopharynx, and larynx and present better prognosis in terms of RFS; in the stromal compartment, a high number of LC is also associated with better prognosis in terms of both RFS and OS. Thus, LC infiltration is a strong and independent prognostic marker for this type of tumor in relation to RFS and OS (76). Decreased ITM number of LC is observed in patients showing HPV-positive head and neck tumor or dysplasia when compared to HPV-negative patients, suggesting that HPV may somehow impair the LC recruitment.

Macrophages. The diversity of data regarding this cell in HPV-tumors is great and somewhat contrasting. This cell population in cervical tissue was first investigated by Tay et al. (1987), showing a strong density in HPV infection and being decreased both in the stroma and in the epithelium with increasing severity of CIN. Although not finding any association between macrophage density and prognosis, Hachisuga et al. (1989) corroborated the Tay et al. finding, demonstrating that macrophages density in the edge of lesions do not differ between progressive grades of CIN.

However, it was observed a great staining for lysozyme-positive tumor-associated macrophages (TAMs) in invasive SCC with strong stromal lymphoid infiltration, suggesting these two events are correlated. Indeed, invasive carcinomas have a significantly elevated TAMs $CD68^+$ counts compared to all CIN grades (78).

In agreement with this, Davidson et al. (1999) also showed TAM density is not associated to prognosis and survival in SCC, as well as with other clinicopathologic features as tumor stage, grade or microvessel counts. In invasive SCC, an inverse correlation between tumor stage and

CD68 positivity (i.e., great TAM density in lower staged tumors) and a positive correlation between CD68 staining and lymphovascular space involvement are observed (80).

Inconsonant with the results described above, a relationship between macrophage counts and CIN grade was demonstrated, with significant differences in macrophages $CD68^+$ numbers between all CIN grades and SCC (81). They are more likely to appear in the stroma but may show a switch from the stroma to the epithelium as the CIN progresses. TAMs $CD68^+$ are found relatively in high number infiltrating the primary tumor and cervical metastases, regardless of HLA class I and II expression, with a trend to decrease in metastatic lesions (82).

Otherwise, TAMs $CD16^+$ are positively correlated with HLA-DR expression in CC (83). TAMs $CD68^+$ density is related to monocyte-chemo-attractant protein-1 (MCP-1) expression in SCC and ADC, appearing immediately adjacent to MCP-1 gene expression at the stroma-carcinoma-cell junction (84).

TAMs prognostic significance in CC have been elucidated. Evaluation of TAMs $CD163^+$ immunostaining in SCC FIGO IB and IIA patients has shown a significant association of high number of peritumorally localized TAMs with short 5-year RFS and lymph node metastasis (38). However, PTM TAM density was not an independent predictor of poor RFS in this cohort. In a comprehensive and refined design, de Vos van Steenwijk et al. (2013) established a robust relation between TAMs subsets infiltration and prognosis in CC analyzing tumor-infiltrating myeloid cells in respect to CD14, CD33 and CD163 expression (i.e. expressed in macrophages, nonterminally differentiated myeloid cells and M2 polarized macrophages, respectively). They have shown interesting results: (i) intraepithelial mature M1 TAMs ($CD14^+$ $CD33^-$ $CD163^-$) are associated with better 5-year disease-specific survival (DSS); (ii) M1 TAMs and intraepithelial T cell infiltration are correlated, especially $CD8^+$ T cells, suggesting they jointly act to an efficient anti-tumor response and improve DSS; (iii) a dense M1 macrophages tumor-epithelial infiltration is an independent prognostic factor for DSS. M2 macrophages are supposed to have a negative impact on cervical cancer therapy, such as therapy with immunoglobulin G (IgG) antibodies directed against epidermal growth factor receptor (EGFR) (86).

TAMs infiltration has also been described in other HPV-associated tumors. Alves et al. (2017) reviewed systematically this relation in OSCC. Altogether, the accumulated data about TAMs infiltration and OSCC show strong evidence toward poor prognosis. In summary, presence of PTM TAMs $CD68^+$ or $CD163^+$ or mannose receptor C type 1^+ (MRC1), stromal $CD68^+$ or $CD163^+$ TAMs in the invasive front of the tumor are all associated with worse prognosis. Analysis of myeloid cell infiltrates in usual-type vulvar intraepithelial neoplasia (uVIN) lesions and

vulvar carcinoma (VC) showed stromal M2 $CD163^+$ TAMs are more present in the progressive course of uVIN, but epithelial M1 $CD14^+$ $CD163^-$ TAMs were increased in VC (88). Multivariate analysis showed a high number of intraepithelial $CD14^+$ TAMs (irrespective of M1 or M2 type) in uVIN is an independent prognostic factor for short RFS; a high number of intraepithelial $CD14^+$ TAMs is also associated with intraepithelial regulatory T cells (Treg) infiltration and lower numbers of stromal T $CD8^+$ cells. Tregs are overall associated with poorer prognosis in human cancers, while T $CD8^+$ cells infiltration has a favorable prognostic impact due to its anti-tumor effects (89,90).

Regarding anal cancer, CD68 immunostaining demonstrated stromal TAMs infiltration progressively increase as the pre-invasive dysplastic lesions progress to anal intraepithelial neoplasia to anal squamous cell carcinoma (ASCC). Moreover, TAMs infiltration was positively correlated with VEGF expression and MVD, again drawing attention to the relationship between macrophages and angiogenesis (91).

Natural Killer cells. All grades of CIN demonstrated a positive immunostaining for natural killer (NK) cells, with a similar pattern of density, usually small and predominantly being found in subepithelial stroma than intraepithelial tissue (92). A larger sampling showed NK cells infiltration in the stroma and the epithelium are significantly increased in CIN 2/3 compared with the normal cervix (36).

NK cells were virtually non-existent in CC specimens, given its paucity in both normal and tumor tissues (93). Piersma et al. (2007) also described small counts of $CD57^+$ NK cells in cervical tumors. Hachisuga et al. (2001) verified no association between $CD57^+$ NK cells presence and clinicopathologic features and prognosis in SCC patients. However, the number of NK cells was correlated with LC presence.

Although NK cells density is minor in comparison to other immune cells, this result suggests a role for NK cells in anti-tumor localized immune response. In fact, $CD56^+$ NK cells infiltrate the tumor, and HLA class II molecules may play a role in this phenomenon in CC (83).

$CD56^+$ NK cells number are elevated in the primary tumor of HNSCC patients in response to IL-12 treatment (95). These patients have decreased serum levels of IL-12, which would impair NK cells infiltration (96).

Data about the prognostic value of NK infiltration cells in this type of cancer is scarce, but patients with a high infiltration of NK cells showed significant correlation with patient survival

(97). Elevated NK lytic activity have a favorable prognosis demonstrated by an improved DFS (98).

Primary tumors and lymph node metastases from vulvar squamous cell carcinoma (vSCC) patients were analyzed regarding $CD56^+$ NK cells and lymphocytes expressing granzyme B (GrB^+) (i.e., $CD8^+$ cells and NK/NKT cells) infiltrates (99). NK cells were detected in ITM region and sporadically in the mesenchymal stroma, while GrB^+ cells predominantly infiltrate the tumor stroma. Analyzing the prognostic significance of these cell populations, high intraepithelial GrB^+ infiltrates predicted improved OS in patients with local tumors; high intraepithelial $CD56^+$ predicts longer OS in metastatic ones.

Another subset of T cells, the type I invariant natural killer T (iNKT) cell (i.e., it express the semi-invariant T cell receptor), has a role in tumor immune surveillance and presents phenotypic and functional similarities with NK cells (100). However, iNKT cell cytotoxicity is mostly restricted to the CD95/CD178 pathway rather than perforin/granzyme-mediated mechanisms.

Interestingly, there is a higher proportion of infiltrating iNKT cells in persistently hrHPV-infected cervical biopsies, mainly in CIN III (101). Despite preclinical models indicate an antitumor activity displayed by iNKT cells (102), this result suggests iNKT cells may play a regulatory role in cervical carcinogenesis.

Otherwise, in a longitudinal cohort, low numbers of circulating iNKT were independently associated with decreased 3-years OS and LCR in HNSCC patients before treatment, demonstrating iNKT cells deficiency causes a poor clinical outcome in response to radiotherapy (103). Circulating NK cells were also reduced in these patients, but no correlation with prognosis was found.

B cells. The assumption that humoral immune response participates in tumor immune surveillance is not a novelty, as demonstrated by McCoy et al. (1979) when they showed the presence of tumor-bound immunoglobulin (Ig) G in gynecologic tumors, especially CC.

B cells $CD19^+$ are found infiltrating CC, but flow cytometry analysis has shown their number in ADC is lower in comparison with SCC specimens, indicating a protective role of high B cell levels in SCC (105). Higher numbers of B cells $CD19^+$ are observed in lymph nodes when compared to peripheral blood and cervical tissue of CC patients with stage IB–IIA before therapy, leading us to think tumor antigens-driven B cell responses take place primarily at regional lymph nodes in CC (106).

CIN patients show no difference in B cells $CD19^+$ counts in relation to controls in cervical tissue and peripheral blood (107). In contrast with this, LSIL show IgG-positive plasma cells infiltrating subepithelial stroma to a greater extent than HSIL or ICC; B cells are also found in lesions that have progressed to CC, but in a lesser extent than in SIL (108). It is not difficult to rationalize the great recruitment of B cells to precursor lesions rather than cervical tumor may be a reflection of a humoral immune response to active HPV infection (109).

In this sense, Feng et al. (2012) found strong evidence of B cells involvement in CC. They quantified the infiltrating $CD20^+$, $CD138^+$, and $CD32B^+$ lymphoid cells (i.e., B, plasma and Fc receptor-expressing cells markers, respectively) in cervical stroma and they were all correlated; $CD20^+$ and $CD138^+$ numbers were increased in CIN 1, while $CD138^+$ and $CD32B^+$ were elevated in ICC in comparison to HSIL and CIS. Furthermore, B cells infiltration were associated with T_{h2} inflammation and tumor progression; $CD32B^+$ lymphoid cells were associated with tolerogenic markers as IDO 1, IL-10 and forkhead box p3 (FOXP3) expression. B cells $CD20^+$ positive immunostaining is positively correlated with high 5-years survival, decreased risk of relapse and FIGO clinical stage in HPV^+ CC (111).

B cells infiltrates are negligible in HNSCC sections either in ITM or PTM regions (112). However, Chen et al. (2018) found that enhanced infiltration of B cells is an independent protective factor for HPV^+ HNSCC. B cells have been generally overlooked in terms of others HPV-associated tumors, and its effective role in HPV-induced oncogenesis is far from clear.

Cytotoxic T lymphocytes. In an *in vitro* approach used to map cytotoxic T lymphocytes (CTLs) $CD8^+$ responses to HPV-E6 and E7 peptides in different anatomical sites of ICC patients, Evans et al. (1997) revealed for the first time antigen-specific memory CTLs are present in peripheral blood, but are enriched in draining lymph nodes and within tumor. Similar observations were noted in experiments performed in patients with precursor lesions, demonstrating the occurrence of HPV-specific memory CTLs in most of CIN III patients (114).

In accordance, CTLs $CD8^+$ numbers infiltrating dysplastic tissue of cervix is elevated (107). In fact, CTLs $CD8^+$ are the predominant subset observed in LSIL and HSIL, mainly in stroma than the ITM compartment (115). CTLs density is 1.6 fold higher in biopsies of women who presented SIL regression, whereas in persistent lesions this number is 3-fold lower. Similarly, intraepidermal infiltrates of HPV-specific $CD8^+$ CTLs mediate regression of VIN 3 lesions, but $CD8^+$ TILs were found not to be related to prognosis in vSCC (116,117).

ITM $CD8^+$ cells and a higher CD8/CD4 ratio are related to the absence of lymph node metastases in patients with early-stage CC (11). However, a reversed CD4/CD8 ratio and an increased proportion of infiltrating CTLs $CD8^+$ in bulky cervical tumors (i.e., tumor size >4cm) and patients presenting lymph node metastasis have been reported. They may be inefficient in mediating death of malignant cells, once their action is amplified by $CD4^+$ cells (105). Otherwise, IFN-ɣ and IL-2-producing T $CD8^+$ cells predominate in tumor tissues of patients with stage IB–IIA CC and may play a role in the tumor containment (106). Lesser ITM CTLs $CD8^+$ cells infiltration predicts worse survival in ADC. Furthermore, the stronger the COX-2 expression of the tumor, the lesser the ITM CTLs infiltration, highlighting the antagonist role of COX-2 in host immune reactivity against neoplastic cells (118).

Recently, Komdeur et al. (2017) not found a significant association of *CD8A* gene expression alone with prognosis in ICC. However, pushing tumors present a large staining for CD103 (i.e., an epithelial T cell biomarker) and ITM CD8 co-expression than in stromal compartment. Desmoplastic tumor type also contains a high percentage of $CD8^+$ $CD103^+$ TIL. These results demonstrate that $CD103^+$ cells in CC tissue are predominantly $CD8^+$ T cells; besides, these cells were strongly associated with a better predictor of prognosis in patients receiving radiotherapy. Tumor infiltration of $CD8^+$ CTLs has been shown to be valuable in predict favorable clinical outcomes in HNSCC (120). A large cohort revealed that high ITM $CD8^+$ CTLs numbers are associated with better 3-year OS and 3-year DFS in patients carrying tonsillar and base of tongue squamous cell carcinoma (TSCC and BOTSCC, respectively) positive to HPV-DNA and $p16^{INK4a+}$, a marker of active HPV-expressing E7 mRNA (121).

High CD8+ counts in the PTM tissue were also favorable prognostic factors of 5-year OS and 5-year DSS rates in TSCC patients after chemoradiotherapy, being an additional indicator of a successful treatment (122). On the other hand, ITM GrB^+ CTLs infiltration is a negative prognostic factor in patients with ASCC after chemoradiotherapy, suggesting the state of activation rather than the mere presence of CTLs in the tumor microenvironment is important to drive prognosis (123).

T cells. An extensive literature has stated that TIL presence is the best immune prognostic predictor in a high variety of cancers (10). There is a consensus that quantification of CD3/CD45RO, CD3/CD8 or CD8/CD45RO in CT and IM provides a clinically helpful biomarker of clinical outcome, especially in colorectal cancer, but with the potential to be extrapolated to other tumors (124).

Indeed, the prognostic value of $CD3^+$ TIL has already been reported in CC (125). High density of ITM $CD3^+$ cells improved 5-year survival in women bearing early-stage ICC in comparison to those with low $CD3^+$ TIL infiltration. Decreased infiltration of these cells was associated with risk of pelvic lymph node spread and subsequent local or distant disease control failure. High CD4/CD8 ratio is also a prognostic indicator in CC, improving the 5-year survival rate (126). In fact, lower CD4/CD8 ratio and CD4 counts are noted in patients who died of the disease. $CD4^+$ cells predominate in stroma and epithelium of hrHPV-cervical lesions, and the CD4/CD8 ratio is elevated in regressing CIN 1 when compared with CIN 1, CIN 3 and ICC (127). $CD4^+$ cells presence also is correlated with regression in VIN 3 (116). No prognostic significance was found between $CD4^+$ TILs and survival rates in vSCC (117). T cells may also contribute with therapy sensitivity. Activated $CD4^+$ and $CD8^+$ T cells ($CD69^+$) significantly increase over time in locally advanced CC treated with chemoradiation (70). $CD8^+$ granzyme B^+ cells also show the same behavior.

ITM $CD3^+$ T cells are a negative prognostic marker in ASCC, and $CD4^+$ T cells are related to poor prognosis (123). Furthermore, reactive memory $CD45RO^+$ cells are found mainly in stromal compartment than ITM region in ICC, being present in higher numbers than CIN 1 (127). These cells possibly present a regulatory phenotype and induce tumor immune tolerance, contributing to cancer progression (128). However, CD45 immunostaining were correlated with good prognostic factors as high 5-years survival, decreased risk of relapse and FIGO clinical stage in HPV^+ CC (111). Lower densities of PTM CD45RO cells were found among patients with relapse compared to patients without in stage IB SCC (129).

$CD3^+/CD4^+$ T cells are plastic and differentiate into T helper (T_h) populations depending on tumor type and tumor microenvironment. Thus, these cell subsets with different functional activities impact on clinical outcome and should be taken into account when the prognostic evaluation is performed (10).

$CD4^+$ TIL effector cells constitutively express basal levels of both IFN-ɣ and IL-4, a reflex of both T_h1 and T_h2 subsets recruitment in CC (130). Santin et al. (2001) detected a great proportion of IFN-$ɣ^+$ IL-2^+ T_h1 cells in comparison to IL-4^+ T_h2 cells in the tumor, lymph nodes and peripheral blood of early-stage CC patients. Before that, low ITM IFN-ɣ mRNA expression had already been associated with poor prognosis in ICC (131).

In agreement, intraepithelial infiltration of $CD4^+$ and T-bet^+ (i.e., a marker of the T_h1 subset) IFN-γ-producing cells in HSIL predicts a favorable prognosis (132). There is evidence that T_h responses in CC are T_h2-dominant both in stroma and CT when compared with normal tissue

and SIL, verified by biopsies sections with co-localized staining of anti-IL-4 and anti-CD3 mAbs (133). In fact, T_h2 type inflammation is accountable by a gradual progression of CC, and TILs present mainly T_h2/Tc2 (i.e., T cytotoxic-2) phenotypes (110,134).

The overall immunological profile associated with a good prognosis in SIL and ICC is an increased local production of T_h1 cytokines, while T_h2 cytokines are related to worse outcomes (135). Regulatory cytokines expression (i.e., IL-10, TGF-β1, and TGF-β2) is strongly associated with progressive CC, suggesting the Treg infiltration in tumor nests suppress the anti-tumor cellular immunity (135). High $CD4^+$ $FOXP3^+$ Treg cells densities is related to a lower 5-year survival rate in FIGO stage IIB and IIIB SCC patients (126).

T_h17 cells were also reported to be involved in HPV-tumors. CC and SIL patients display a high proportion of $CD4^+$ $IL\text{-}17^+$ TILs, potentially promoting tumor growth and angiogenesis (136). Otherwise, Punt et al. (2015) found an independent association of $CD3^+$ $IL\text{-}17^+$ cells with improved DSS in SCC. T_h17 cells recruitment is a general feature of human cancers and its pro- versus anti-tumoral effects are still a dilemma (137).

Regarding oral cancer, high counts of $CD4^+$ TILs did not influence on TSCC and BOTSCC clinical outcomes (121). Expression of the CD3 ζ-chain is an independent biomarker of survival in the advanced OSCC (138). Intriguingly, $CD4^+$ $FOXP3^+$ was a prognostic factor for LCR, pointing out a role of Tregs in ‘’cooling-down’’ the pro-tumor inflammatory reaction. Treg cells infiltration in HNSCC patients is associated with improved survival after treatment, suggesting the oncologic therapy favors expansion of Treg (139).

$CXCR3^+$ $CCR5^+$ T_h1 cells predominantly infiltrate the subepithelial stroma of oral leukoplakia (i.e., an oral premalignant lesion). These cells express IFN-inducible gene products STAT1 and CXCL9 and induce TAMs polarization towards to M1 phenotype, a mechanism that in this case may be detrimental by generating resistance to the anti-tumor immune responses through IFN-induced cancer immunoediting (140).

In addition, a higher circulatory number of T_h22, T_h17, and T_h1 cells was reported in CC patients (141). T_h22 subset was also correlated with lymph node metastases in these patients. If this cell population is present in the microenvironment of HPV-tumors is still unclear. The role of T_h22 cells in cancer is elusive, but studies show they apparently are deleterious in tumor pathogenesis mainly by IL-22 secretion, known to be a promoter of cell proliferation (142).

Concluding Remarks

Understanding the immune signature importance in cancer outcome, an international task force composed of expert pathologists and immunologists worldwide was created to validate and implement Immunoscore in routine testing. Immunoscore is a prognostic tool designated TNM-I (TNM-Immune) based on the numeration of two lymphocyte populations (cytotoxic and memory T cells) in the center of the tumor (CT), invasive margin (IM) and tertiary lymphoid structures (TLS), and has a prognostic significance superior to that of the AJCC/UICC (American Joint Committee on Cancer and Union for International Cancer Control, respectively) TNM classification system in predicting cancer recurrence (124).

Although these professionals agree that cytotoxic memory $CD8^+$ T cells ($CD3^+$, $CD8^+$, $CD45RO^+$, granzyme B^+) provide the best method to evaluate patient clinical outcome in CT and IM of tumors, technical difficulties in staining CD45RO and granzyme B lead to acceptance of combined CD3 and CD8 staining employment as standard clinical practice (124). Generally, high densities of CD3 and CD8 positive cells predict good clinical outcomes in HPV-related cancers, but it might differ according to the cancer type, as seen in anal cancer.

Both innate and adaptive immune cells are found within or around the tumor, and the immune contexture in HPV-associated tumors have been characterized. Immune cells infiltrates may present heterogeneous profiles between tumor types and vary from patient to patient and stages, sometimes showing conflicting prognostic values (Figure 1 - a,b). It demands a careful interpretation of clinical data regarding cancer outcome, once immune cells are known to have dual roles in tumor microenvironment, displaying both tumor-antagonizing and tumor-promoting properties in most, if not all, cancers. Hanahan and Weinberg (2011) caught this counterintuitive action of immune surveillance, and classified the "tumor-promoting inflammation'' as a hallmark of cancer, posteriorly adding "avoiding immune destruction'' as an emerging hallmark (1).

The establishment of these concepts reflects the successful efforts of scientists in elucidating the underlying cellular and molecular mechanisms of host-tumor interplay. We highlight the advent of immunotherapy discovery with the use of monoclonal antibodies, cell-based therapies and vaccines, which have the power of potentiating the cytotoxic and anti-tumor immunity or blockade inhibitory immune checkpoints. Despite these notable breakthroughs, the establishment of more reliable prognostic biomarkers capable of improve patients survival and avoid

tumor recurrence is indispensable. Considering that the immune cells infiltration in HPV-related cancers influence the natural history of cancer and is a strong prognostic factor to predict disease outcome and response to therapy, we strongly advise that the routine use of Immunoscore in HPV-cancers risk stratification must not be neglected.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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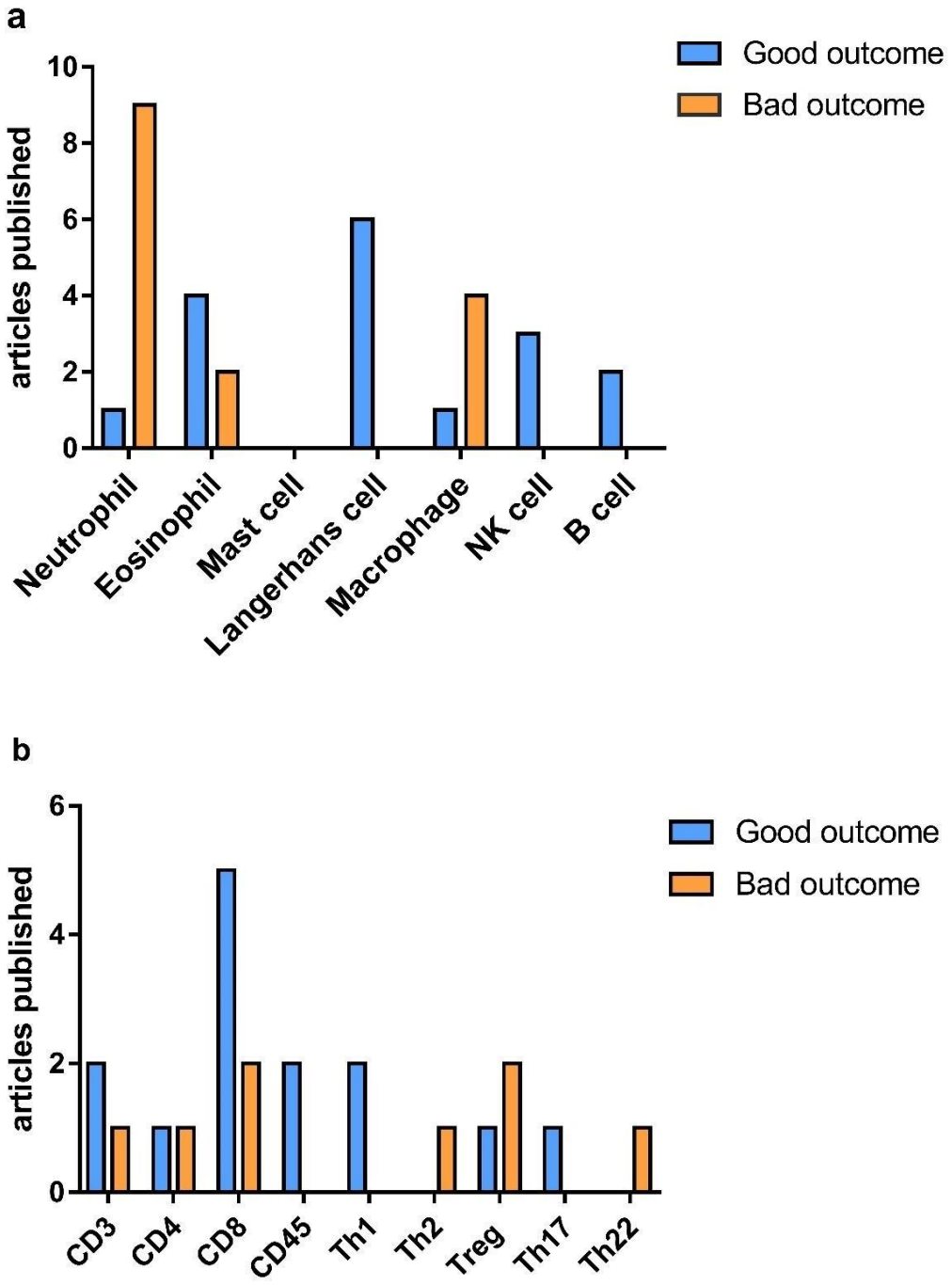

Figure 1. Immune cells involvement in HPV-associated cancers prognosis.

Graphics depict articles revisited in which immune cells infiltration or systemic levels were significantly associated to good or bad outcomes by statistical survival model analysis in HPV-associated cancers in general. Articles that found associations with clinicopathological parameters were excluded. **a)** innate and B cells are shown. **b)** T cells standard markers and T cells subsets subsets are shown.

6 CONCLUSÕES

Artigo 1

✓ A mediana de idade entre pacientes infectadas pelo HPV e portadoras de LSIL foi significativamente menor em relação aos controles.

✓ A detecção do DNA-HPV foi significativamente mais frequente em mulheres com menos de 24 anos, que recebiam menos de 1 salário mínimo, solteiras, tabagistas, nulíparas, cuja sexarca ocorreu antes dos 17 anos e que tiveram mais de 4 parceiros sexuais durante a vida.

✓ Neste delineamento, não encontramos associações significativas entre variáveis sociodemográficas e tipo de HPV (baixo ou alto risco).

✓ Associações positivas foram detectadas entre diagnóstico de LSIL e HSIL e mulheres com menos de 24 anos. Diagnóstico de LSIL esteve associado ao uso de anticoncepcionais hormonais e aborto. Diagnóstico de HSIL esteve associado à baixa renda (menos de 1 salário mínimo), tabagismo e múltiplos parceiros (mais de 4 parceiros sexuais durante a vida).

✓ A presença do alelo C do polimorfismo de *FOXP3* rs3761548 foi positivamente associada ao risco de se contrair a infecção pelo HPV.

✓ O genótipo homozigoto A/A do polimorfismo de *FOXP3* rs3761548 foi independentemente associado à proteção à infecção pelo HPV e desenvolvimento de HSIL.

✓ O genótipo homozigoto G/G do polimorfismo de *FOXP3* rs2232365 foi independentemente associado ao risco de se contrair a infecção pelo HPV.

✓ Os polimorfismos de *FOXP3* não estiveram associados ao genótipo de HPV neste estudo.

✓ A presença do alelo A do polimorfismo de *FOXP3* rs3761548 foi associada com proteção ao desenvolvimento de lesões.

✓ O genótipo homozigoto A/A do polimorfismo de *FOXP3* rs3761548 foi independentemente associado à proteção ao desenvolvimento de HSIL.

✓ O genótipo heterozigoto C/A foi independentemente associado ao risco para o desenvolvimento de HSIL.

✓ Não houve associações positivas entre haplótipos de *FOXP3* e risco de infecção pelo HPV ou diagnóstico de SIL.

Artigo 2

✓ Não houve diferenças estatisticamente significativas entre os níveis plasmáticos e cervicais de IL-10 em mulheres HPV positivas considerando os diferentes modelos de herança genotípica dos polimorfismos de *FOXP3* rs3761548 e rs2232365.

Artigo 3

✓ Quando avaliados em conjunto, os trabalhos publicados na literatura usando modelos estatísticos de sobrevida em cânceres causados por HPV demonstram que estão associadas à bom prognóstico as seguintes células: células de Langerhans; célula NK; célula B; células $CD3^+$, $CD8^+$, $CD45RO^+$, células T_h1 e T_h17.

✓ Células $CD4^+$ conferem bom prognóstico, mas podem estar relacionadas à mau prognóstico em casos específicos, a depender de sua diferenciação em diferentes subpopulações.

✓ Neutrófilo, macrófago, células T_h2, T_h22 e Treg estão associadas à mau prognóstico.

✓ Eosinófilos possuem dados controversos e difíceis de serem generalizados.

✓ Mastócitos não estão associados à prognóstico, mas à eventos patológicos como angiogênese.

7 CONSIDERAÇÕES FINAIS

O câncer de colo de útero tem sido alvo de intensa investigação científica nas últimas décadas, tanto em aspectos da biologia molecular da célula tumoral quanto do ambiente que a circunda. Neste contexto, indubitavelmente, células imunes são as protagonistas e modulam a forma como o hospedeiro responde à presença do tumor. Essa modulação é dependente da produção de moléculas que possuem ação citotóxica direta ou indireta, através do recrutamento de outras células com essa capacidade, ou por meio de mecanismos que ‘‘esfriam’’ a resposta imune. Destacam-se entre essas moléculas citocinas de respostas efetoras antitumorais/antivirais (e.g., interferons, superfamília do TNF, quimiocinas) e mediadores antinflamatórios (e.g., IL-10, TGF-β e FOXP3).

Como visto neste trabalho, SNPs no gene *FOXP3* podem influenciar na oncogênese cervical associada ao HPV, desempenhando um papel dual, ora protetor, ora deletério, e esta influência muito provavelmente está ligada à modulação da expressão do gene, evento sugerido por outros trabalhos. Este estudo é pioneiro e revela pela primeira vez a associação entre variantes de *FOXP3* e HPV. No entanto, este caminho apenas começa a ser pavimentado, e nossos achados provocam mais perguntas do que respostas:

i) Qual é a influência destes SNPs na expressão gênica e proteica de *FOXP3* em um contexto específico (e.g., HPV-positivas *versus* HPV-negativas; CCU *versus* controles)?

ii) Seus efeitos patológicos são devido à influência na regulação gênica ou se devem a um possível desequilíbrio de ligação (LD) forte com vários outros SNPs de *FOXP3* ainda não investigados?

iii) Estes mecanismos são sinérgicos ou há uma predominância de um em detrimento de outro?

iv) Quais são as consequências funcionais na proteína quando estes SNPs estão presentes em forte LD com outros SNPs de *FOXP3* descritos?

v) SNPs de *FOXP3* alteram sítios de metilação, acessibilidade à cromatina, modificações de histonas ou RNAs *enhancers*?

vi) SNPs de *FOXP3* contribuem com a regulação gênica através de interações 3D?

vii) SNPs de *FOXP3* podem influenciar na expressão gênica, proteica ou tecidual de outros mediadores importantes para a função de células Treg (e.g., citocinas, receptores de citocinas, receptores de *checkpoints* imunes, *clusters*)?

Responder à estas perguntas e determinar a real interferência biológica de um SNP de maneira global não é tarefa fácil, uma vez que as consequências funcionais de uma variante associada à uma doença podem ser complexas e afetar múltiplos genes e vias moleculares, e serem passíveis de um universo de modificações transcricionais e pós-transcricionais que ocorrem no próprio gene afetado e também em genes adjacentes.

Além de técnicas de expressão gênica mais utilizadas como PCR quantitativa, microarranjo, técnicas imuno-histoquímicas e imunoensaios, outras técnicas devem também ser consideradas, como análises *in silico* para predição de sítios de ligação para fatores de transcrição e miRNA, sequenciamento de última geração e espectrometria de massa para identificação de ácidos nucleicos e proteínas que interagem com sítios polimórficos.

Interessantemente, a edição de DNA, notavelmente o CRISPR/Cas9, capaz de editar o sítio de DNA mutado e substituir pela base ancestral, está sendo cada vez mais utilizada para abordar mutações genéticas, especialmente em modelos celulares, mais permissivos para esse tipo de manipulação experimental, mas que podem não refletir o ambiente complexo de células e tecidos do hospedeiro, principalmente quando há tumorigênese.

Abordagens moleculares integradas das variações genéticas já associadas a doenças, com foco no gene *FOXP3*, mas também outros genes imunossupressores, em todos os âmbitos das ciências ômicas, são necessárias para desvendar o papel destas variantes, principalmente as presentes em regiões não traduzíveis, na patogênese viral mediada pelo HPV e nos tumores por ele causados.

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9 ANEXOS

9.1 ANEXO A

Termo de Consentimento Livre e Esclarecido

"Prevalência e genotipagem de HPV e sua possível associação com os genes de citocinas, quimiocinas e seus receptores em nível de DNA, RNA e proteína: implicações no microambiente tumoral."

Prezado(a) Senhor(a):

Gostaríamos de convidá-lo (a) a participar da pesquisa **"Prevalência e genotipagem de HPV e sua possível associação com os genes de citocinas, quimiocinas e seus receptores em nível de DNA, RNA e proteína: implicações no microambiente tumoral."**, realizada no **"Laboratório de Genética Molecular e Imunologia, Departamento de Ciências Patológicas da Universidade Estadual de Londrina".** O objetivo da pesquisa é avaliar a presença do vírus em mulheres atendidas em programas de prevenção ao câncer cervical do setor público de saúde da região norte do Paraná, por meio de metodologia específica e sensível, visando também à associação de dados demográficos, para análise dos fatores de risco que contribuem para a exposição da população ao vírus, bem como os determinantes de sua manutenção. Adicionalmente objetiva-se compreender o papel do sistema imune no controle e iniciação tumoral, bem como na sua formação, crescimento e progressão, em especial avaliar a interação tumor-hospedeiro em pacientes portadoras do vírus HPV e no desenvolvimento do câncer cervical**.** A sua participação é muito importante e ela se daria da seguinte forma: **doação de 5mL de sangue periférico coletado por punção venosa e doação do swab cérvico-vaginal utilizado para confecção das lâminas para o exame preventivo para anállises moleculares, bem como responder um questionário sociodemográfico.** Gostaríamos de esclarecer que sua participação é totalmente voluntária, podendo você: recusar-se a participar, ou mesmo desistir a qualquer momento sem que isto acarrete qualquer ônus ou prejuízo à sua pessoa. Informamos ainda que as informações serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade, de modo a preservar a sua identidade.
As amostras biológicas (sangue periférico e secreção cérvico-vaginal) serão utilizados para extração de DNA e RNA para análises moleculares e imunológicas. Estes materiais serão obtidos em pequenas quantidades portanto não haverá sobra de material biológico.
Os benefícios esperados são a detecção precoce do vírus HPV em mulheres atendidas em programas de prevenção ao câncer de colo de útero do setor público de saúde da região norte do Paraná. Informamos que a paciente que se dispor a participar do projeto não sofrerá desconfortos nem riscos à saúde, não havendo qualquer prejuízo às mesmas. Informamos que a senhora não pagará nem será remunerada por sua participação. Garantimos, no entanto, que todas as despesas decorrentes da pesquisa serão ressarcidas, quando devidas e decorrentes especificamente de sua participação na pesquisa.
Caso você tenha dúvidas ou necessite de maiores esclarecimentos pode nos contactar **Karen Brajão de Oliveira, Laboratório de Genética Molecular e Imunologia, Departamento de Ciências Patológicas, Universidade Estadual de Londrina, 3371-4267, karen.brajao@uel.br),** ou procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 33712490. Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas, devidamente preenchida e assinada entregue a você.

Londrina, ___ de _____de 201__.

Pesquisador Responsável _____
Profª. Drª. Karen Brajão de Oliveira
RG:: 6.538.742-5

_____ (nome por extenso do sujeito de pesquisa), tendo sido devidamente esclarecido sobre os procedimentos da pesquisa, concordo em participar **voluntariamente** da pesquisa descrita acima.

Assinatura (ou impressão dactiloscópica):_____
Data:_____

9.2 ANEXO B

Nº LAB

QUESTIONÁRIO SOCIOEPIDEMIOLÓGICO

Data: ____/____/_____ Reg. N° _____

1. Conhece o HPV???
( ) Nunca ouvi falar
( ) Já ouvi falar mas não sei o que é
( ) Conheço

2. Idade _____ anos DN, _____

3. Etnia: _____
Branca / parda / negra / asiática / indígena

4. Sua renda mensal (em salário mínimo) é de?
( ) Até 1 Salário ( ) De 1 à 3 salários
( ) De 3 à 5 salários ( ) De 5 à 7 salários
( ) De 7 à 10 salários

5. Você fuma?
( ) Não ( ) Sim Tempo: _____

6. Qual o seu grau de escolaridade?
( ) Fundamental Incompleto
( ) Fundamental Completo
( ) Médio Incompleto ( ) Médio completo
( ) Superior incompleto ( ) Sup. completo

7. Estado Civil:
( ) Solteira ( ) Casada
( ) Divorciada ( ) Viúva

8. Qual sua profissão?
_____

9. Faz o uso de algum método contraceptivo?
( ) Não ( ) Sim Qual: _____

10. Tipo de Parto:
( ) Normal ( ) Cesária

11. Nºde gestações: _____

12. Números de Partos:
( ) Nenhum ( ) Um
( ) Dois ( ) Três
( ) Quatro ou mais

13. Idade da 1ª relação sexual: _____anos

14. Idade da 1ª menstruação: _____ anos

15. Número de parceiros sexuais durante a vida:

_____

16. Número de parceiros sexuais nos últimos 6 meses: _____ .

17. Já realizou outros exames preventivos?
( ) Sim ( ) Não

18. Exames de prevenção realizados no passado apresentaram algum tipo de alteração?
( ) Sim ( ) Não
( ) Não me lembro
Em caso de resposta "Sim" favor descrever a alteração: _____

19. Já contraiu alguma infecção ginecológica
( ) Não ( ) Sim ( ) não sei informar
Em caso de resposta ``SIM``, se possível descrever qual:_____

20. Já esteve infectada pelo HPV?
( ) Sim ( ) Não ( ) Não sei informar

21. Conhece as formas de transmissão ou formas de contrair o vírus?
( ) Não ( ) Sim Qual ou quais:
_____

22. Existem casos de câncer de colo de útero em sua família?
( ) Sim ( ) Não
Em caso de resposta ``SIM`` descrever o grau de parentesco: _____

Pesquisador: _____

9.3 ANEXO C

UNIVERSIDADE ESTADUAL DE LONDRINA

PARANÁ
GOVERNO DO ESTADO

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	133/2012
CAAE:	05505912.0.0000.5231
Processo:	19275/2012
Pesquisador(a):	Karen Brajão de Oliveira
Unidade/Órgão:	CCB – Departamento de Ciências Patológicas

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"PREVALÊNCIA E GENOTIPAGEM DE HPV E SUA POSSÍVEL ASSOCIAÇÃO COM OS GENES DE CITOCINAS, QUIMIOCINAS E SEUS RECEPTORES EM NÍVEL DE DNA, RNA E PROTEÍNA: implicações no microambiente tumoral."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá ser encaminhado ao CEP/UEL relatório final da pesquisa, conforme prevê a Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares.

Londrina, 28 de agosto de 2012.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Profª Drª Paula Mariza Zedu Alliprandini
Vice-Coord. do Comitê de Ética em Pesquisa
Envolvendo Seres Humanos
Universidade estadual de Londrina