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PREVALÊNCIA DO PAPILOMAVÍRUS HUMANO E ASSOCIAÇÃO DOS POLIMORFISMOS GST NA INFECÇÃO VIRAL

FRANCISCO BELTRÃO – PR
(NOVEMBRO/2021)

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ANA PAULA REOLON BORTOLLI

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Essa dissertação foi julgada adequada para obtenção do título de Mestre em Ciências Aplicadas à Saúde e aprovada em sua forma final pela Orientadora e pela Banca Examinadora.

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“Procure obter sabedoria e entendimento; não se esqueça das minhas palavras nem delas se afaste.
Não abandone a sabedoria, e ela o protegerá; ame-a, e ela cuidará de você.
O conselho da sabedoria é: procure obter sabedoria; use tudo que você possui para adquirir entendimento.
Dedique alta estima à sabedoria, e ela o exaltará; abrace-a, e ela o honrará.”
Provérbios 4:5-8

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

CC – Câncer Cervical

GST – Glutationas S-Transferase

HPV - Papilomavírus Humano

HSIL - (High grade intraepithelial lesions) Lesões Intraepiteliais Escamosas de alto grau

IST – Infecção Sexualmente Transmissível

LSIL **–** (Low grade intraepithelial lesions) Lesões Escamosas de baixo grau

NIC I - Neoplasia Intraepitelial Cervical grau 1

NIC II – Neoplasia Intraepitelial Cervical grau 2

NIC III - Neoplasia Intraepitelial Cervical grau 3

PCR – Reação em Cadeia da Polimerase

SNP - Polimorfismo de Nucleotídeo Único

GSTM1 - Isoforma μ da Glutationa

GSTT1 - Isoforma θ da Glutationa

ERO - Espécies Reativas de Oxigênio

GSH - Glutationa

PREVALÊNCIA DO PAPILOMAVÍRUS HUMANO E ASSOCIAÇÃO DOS POLIMORFISMOS GST NA INFECÇÃO VIRAL

Resumo

A infecção causada pelo Papilomavírus humano (HPV) é comum em mulheres sexualmente ativas e pode causar lesões intraepiteliais podendo progredir para câncer cervical (CC). O objetivo do estudo foi determinar a prevalência do HPV e investigar se há relação entre os polimorfismos, GSTT1 e GSTM1, com a susceptibilidade para a infecção viral e fatores de risco associados em um grupo de mulheres. A pesquisa envolveu 324 mulheres, que foram recrutadas em serviços da rede credenciada ao Sistema Único de Saúde. As participantes foram entrevistadas e através da coleta do material biológico das escovas endocervicais do exame Papanicolau foi determinado a prevalência do vírus e a caracterização dos polimorfismos GSTM1 e GSTT1. Para detecção molecular do HPV, foram utilizados iniciadores da região codificadora do gene L1 do vírus, MY09 (5'-CGTCCMAARGGAWACTGATC-3') e MY11 (5'-GCMCAGGGWCATAAYAATGG-3'), obtendo um fragmento de 450 pb. O método PCR Multiplex foi usado para determinar os polimorfismos genéticos GSTM1 e GSTT1. Os pares de iniciadores utilizados foram: Forward 5'-GAACTCCCTGAAAAGCTAAAGC-3' e reverse 5'-GTTGGGCTCAAATA TACGGTGG-3' e Forward 5'-TTCCTTACTGGTCCTCACATCTC-3' e reverse 5'-TCACCGGATCATGGCCAGCA-3'. O primeiro produz um amplicon de 219pb e o segundo de 459pb, respectivos, aos genes GSTM1 e GSTT1. O HPV foi detectado em 22 (6,8%) mulheres e destas sete (58,3%) apresentavam alterações citopatológicas. O modelo multivariado apontou como possíveis associações com a presença do HPV as alterações cervicais atuais que ampliam quase 33 vezes a chance da presença do HPV (OR_{adj}: 32.688; IC 95% 8.508-125.589; p<0.001), infecção vaginal recente (OR_{adj}: 2.773; IC 95% 1.048-7.341; p<0.040) e pertencer a outras raças que não a branca (OR_{adj}: 3.058; IC 95% 1.056-8.857; p<0.039). Da amostra inicial foi delineado um estudo caso-controle para verificar a relação entre GSTM1 e GSTT1 com a infecção viral. O número de casos (21) e de controles (84) foram pareados por idade. No estudo a prevalência do HPV foi de 6,8%, sendo que

destas 58,3% apresentavam alterações citopatológicas. E mostrou que o hábito de fumar eleva até 3,6 vezes a chance para infecção viral. Ainda, revelou de forma inédita que o alelo nulo GSTT1 foi fator protetivo, ou seja, as mulheres que possuem a deleção estão menos susceptíveis a infecção por HPV.

Palavras-chave: Papilomavírus Humano; GSTT1; GSTM1; Infecção sexualmente transmissível; mulheres.

PREVALENCE OF HUMAN PAPILLOMAVIRUS AND ASSOCIATION OF GST POLYMORPHISMS IN VIRAL INFECTION

Abstract

The infection caused by Human Papillomavirus (HPV) is common in sexually active women and can cause intraepithelial lesions that can progress to cervical cancer (CC). The objective of the study was to determine the prevalence of HPV and to investigate whether there is a relationship between polymorphisms, GSTT1 and GSTM1, with susceptibility to viral infection and associated risk factors in a group of women. The largest survey involved 324 women, who were recruited in services of the network accredited to the Unified Health System (*Sistema Único de Saúde*). The participants were interviewed and through the collection of biological material from the endocervical brushes of the Pap smear, the prevalence of the virus and the characterization of GSTM1 and GSTT1 polymorphisms were determined. For molecular detection of HPV, primers from the coding region of the virus L1 gene were used, MY09 (5'-CGTCCMAARGGAWACTGATC-3') and MY11 (5'-GCMCAGGGWCATAAYAATGG-3'), obtaining a 450 bp fragment. The Multiplex PCR method was used to determine the GSTM1 and GSTT1 genetic polymorphisms. The primer pairs used were: Forward 5'-GAACTCCCTGAAAAGCTAAAGC-3' and reverse 5'-GTTGGGCTCAAATA TACGGTGG-3' and Forward 5'-TTCCTTACTGGTCCTCACATCTC-3' and reverse 5'-TCACCGGATCATGGCCAGCA-3'. The first produces an amplicon of 219bp and the second of 459bp, respectively, for the GSTM1 and GSTT1 genes. HPV was detected in 22 (6.8%) women and of these seven (58.3%) had cytopathological alterations. The multivariate model pointed out as possible associations with the presence of HPV the current cervical changes that increase almost 33 times the chance of the presence of HPV (OR_{adj}: 32.688; CI 95% 8.508-125.589; p<0.001), recent vaginal infection (OR_{adj}: 2.773; 95% CI 1.048-7.341; p<0.040) and belong to races other than white (OR_{adj}: 3.058; 95% CI 1.056-8.857; p<0.039). A case-control study was designed from the initial sample to verify the relationship between GSTM1 and GSTT1 with viral infection. The number of cases (21) and controls (84) were matched by age. In the study, the prevalence of HPV was 6.8%, and these 58.3% had cytopathological changes. Smoking has been shown to increase the chance of

viral infection up to 3.6 times. Still, it revealed in an unprecedented way that the GSTT1 null allele was a protective factor, that is, because women who have a deletion are less susceptible to HPV infection.

Keywords: Human Papillomavirus; GSTT1; GSTM1; Sexually Transmitted Disease; women.

SUMÁRIO

1. INTRODUÇÃO GERAL

O Papilomavírus Humano (HPV) é capaz de infectar tanto a pele quanto as mucosas e reúne mais de 200 subtipos distintos (TUMBAN, 2019). Destes, 40 possuem potencial para infectar o trato anogenital, sendo reconhecido como causador da infecção sexualmente transmissível (IST) mais comum no mundo (COSER et al., 2016). Morfologicamente, é um vírus pequeno (cerca de 55 nm), com estrutura simples, não envelopado, de formato icosaédrico, constituído por dupla fita de DNA circular, protegida pelo capsídeo formado por 72 capsômeros NAKAGAWA et al., 2010; LETO et al., 2011; FIGUEREDO et al., 2013; KNIPE e HOWLEY, 2013). O genoma do HPV possui entre 6800 e 8400 pares de bases (pb) (SENBA, MORI, 2012). Possui oito regiões conhecidas como fases de leitura aberta (Open Reading Frames) e uma região não-codificadora. As fases de leitura aberta são organizadas em três regiões: a regulatória (LCR - log control region) ou URR (upstream regulatory region), a precoce (Early – genes E1 a E7) e a tardia (Late – L1 e L2) (MUÑOZ et al., 2006; BROWN et al., 2008; SANTOS-LÓPEZ et al., 2015).

A diferença entre os tipos de HPV encontrados em tumores benignos e malignos permite classificá-los como de baixo e alto risco oncogênico (FRANCO et al., 2001; LETO et al., 2011). Dentre os subtipos, 12 são reconhecidos como de alto risco para o desenvolvimento do câncer cervical (CC), especialmente os 16 e 18 (CHATURVEDI, 2010; GUAN et al., 2012; LOWY e SCHILLER, 2012; CROSBIE et al., 2013; PATEL et al., 2013; PLUMMER et al., 2016; BRAY et al., 2018; SUK et al., 2018; VAN DYNE et al., 2018; BRUNI et al., 2019). Entretanto, o CC possui bom prognóstico se descoberto e tratado precocemente (PAL e KUNDU, 2020; ZHU et al., 2020).

A transmissão do HPV acontece por contato direto com a pele ou mucosa, principalmente durante a relação sexual e pode ocorrer em uma única exposição. O contágio pode ser oral-genital, genital-genital, anogenital ou mesmo manual-genital, logo, não há obrigatoriedade de penetração vaginal ou anal (INSTITUTO DO HPV, 2013; BRASIL, 2020). Normalmente, estas células são expostas ao vírus através da abrasão e consequente formação de microlesões na mucosa durante a relação sexual (SCHÄFER et al., 2015). O risco geral estimado para a exposição a essa infecção a cada nova parceria sexual é de 15% a 25% e quase todos os

indivíduos sexualmente ativos adquirirá a infecção em algum momento da vida (WINER et al., 2008). Segundo Doorbar (2005), o ciclo normal da infecção pelo HPV passa por cinco etapas que incluem: infecção, manutenção do genoma, fase proliferativa, amplificação genômica e, por fim, síntese e liberação de novas partículas virais. Ainda, a transmissão do vírus pode se efetivar durante o parto com a formação de lesões cutaneomucosas em recém-nascidos, ou podendo progredir para a manifestação papilomatose recorrente de laringe (BRASIL, 2020).

Estatísticas globais revelam que o CC é o quarto câncer mais frequentemente diagnosticado e a quarta causa de morte por câncer em mulheres, com uma estimativa de 604.000 novos casos e 342.000 mortes em todo o mundo em 2020 (SUNG et al., 2020). O número de óbitos por esta doença no estado do Paraná em 2019 foi de 336 mulheres (SESA/PR, 2020). De acordo com o Instituto Nacional do Câncer (INCA), para cada ano do triênio 2020-2022, a estimativa de novos casos no Brasil é de 16.590, destes, 990 no estado do Paraná (MS/INCA, 2020). Embora, a infecção pelo HPV seja o principal fator de risco para o CC, sozinha, é na maioria das vezes um evento insuficiente (SCHIFFMAN et al., 2007). Além disso, o vírus pode ser detectado em mulheres com citologia normal e nem todas as infectadas irão apresentar lesões no colo uterino e o câncer (AYRES e SILVA, 2010; COLPANI et al., 2016).

Fatores que desempenham um papel importante na biologia viral e progressão da doença estão bem definidos na literatura, tais como: início precoce da atividade sexual (CASTELLSAGUÉ et al., 2002; MELO et al., 2019), raça (WENDLAND et al., 2020), uso de anticoncepcionais (GADDUCCI et al., 2011; IVERSEN et al., 2021), exposição ao tabaco (SIOKOS et al., 2019; WENDLAND et al., 2020), coinfecção por mais de um subtipo viral (LOPÔ, 2006; STANLEY, 2012), o estresse oxidativo (CRUZ-GREGORIO et al., 2018), fatores imunológicos e genéticos (PUDNEY et al., 2005).

Os organismos estão continuamente expostos a agentes químicos estranhos ao sistema biológico como fármacos, tabaco, álcool, aditivos alimentares, agentes poluentes, pesticidas, entre outros, denominados de xenobióticos. Estes são metabolizados nas células e submetidos a um processo de detoxificação enzimática que engloba três fases distintas. Nas fases I e II, acontece a transformação do xenobiótico em uma espécie que apresenta maior solubilidade em água e menor toxicidade (HAYES et al., 2005; CHEN, 2012). Na fase III, estes

metabólitos são transportados para o exterior da célula e então excretados. Especialmente, na fase II da detoxificação há ação de um grupo importante de proteínas, as glutationas (GSH) (REBBECK, 1997; SEIDEGARD et al., 1999; HUBER et al., 2008). Estas são enzimas que participam do processo de desintoxicação de uma variedade de xenobióticos, por vias não-enzimática ou por conjugação enzimática de metabólitos catalisada pela glutationa-S-transferase (GST). As GSTs são divididas em duas superfamílias distintas, a microssomal e a citosólica. As GSTs citosólicas estão distribuídas em oito classes gênicas: alfa (α), mu (μ), Pi (π), Theta (θ), sigma (σ), Kappa (κ), Omega (ω) e Zeta (ζ) (MANNERVIK et al., 1992; TOWNSEND e TEW, 2003; HOLLMAN et al., 2016; CHATTERJEE e GUPTA, 2018). Todas essas classes apresentam polimorfismos genéticos populacionais, dentre eles, os de nucleotídeo único (SNP). Um SNP presente no éxon de um gene, pode ser responsável pela incorporação de um aminoácido alternativo, provocando inúmeros alterações e distúrbios genéticos a depender do gene afetado. Especificamente, os SNPs que provocam deleções têm uma profunda influência na estrutura e interação das proteínas, tendo em vista que o alelo nulo significa ausência ou comprometimento da função proteica. Assim, identificar e caracterizar os SNPs de deleção é uma questão importante para ser compreendida (ZOU et al., 2016; KENNEY et al., 2017).

A possibilidade de reconhecer diferenças genéticas inofensivas ou que resultam em alterações na função gênica, como o polimorfismo de nucleotídeo único não sinônimo (nsSNP) ou mutações raras (missense), é de suma importância para a diagnóstico precoce de pacientes com risco de desenvolver patologias específicas, abrindo caminho para o diagnóstico personalizado (KUCUKKAL et al., 2014; YADAV et al., 2014).

A glutationa- S-transferase (GST) constitui um grupo de enzimas que atua na fase II do metabolismo de xenobióticos, conjugando os derivados metabólitos com a glutationa, tornando-os mais solúveis em água, consequentemente, reduzindo o efeito citotóxico (HAYES et al., 2005). Os genes responsáveis por codificar as enzimas, glutationas S-transferases (GST), ganharam destaque nas últimas décadas na área de oncogenética. Sobretudo, os polimorfismos gênicos das GST têm despertado interesse em pesquisas de diversos tipos de câncer (TOWNSEND e TEW, 2003) dentre eles, o câncer cervical.

Os genes GSTM1 localizado no cromossomo 1p13.3 e o GSTT1 em 22q11.23, pertencem, respectivamente, as classes Mu e Theta (TEW et al., 2011). Os polimorfismos genéticos classificados como nulos são resultantes de deleções gênicas (LUO et al., 2011). Nesse caso, os genótipos nulos para GST levam a incapacidade de produzir a variante proteica, provocando perda da ação delas durante a detoxificação dos xenobióticos (TOWNSEND e TEW, 2003; TATEWAKI et al., 2009; HOLLMAN et al., 2016). As variações alélicas, resultantes de SNPs, nas GSTs têm apresentado associação com patologias como diabetes mellitus e diferentes tipos de câncer (HAYES e STRANGE, 2000; KUCUKKAL et al., 2014; AFRAND et al., 2015; MCILWAIN et al., 2006; STOJKOVIC LALOSEVIC et al., 2019). Logo, sugere o uso dos SNPs como biomarcador genético individual ou populacional para o risco de algumas patologias (HOLLMAN et al., 2016). Indivíduos com genótipo nulo, GSTM1 e GSTT1, correspondem a 50% e 18% da população branca, respectivamente. Esse perfil genético, por não conjugar os metabólicos da fase II do metabolismo de xenobióticos, pode ser mais susceptível aos danos causados pelo estresse oxidativo e, talvez às infecções virais (EATON e BLAMMLER 1999; LANDI, 2000).

Há vários estudos que apontam para uma relação entre os genótipos nulos GSTM1 e GSTT1 e o câncer de colo de útero (KIM et al., 2000; SINGH et al., 2008; GAO et al., 2011; SHARMA et al., 2015). Contudo, há controvérsias entre as populações, restringindo a generalização de tal associação (SHARMA et al. 2004; SOBTI et al., 2008; STOSIC et al., 2014). Nesse contexto, a maioria dos trabalhos priorizam conhecer a relação entre os alelos, GSTM1 e GSTT1, e as lesões intraepiteliais e a neoplasia cervical (ANANTHARAMAN et al., 2007; PALMA et al., 2010; SHARMA et al., 2015), sendo pouco explorado a relação entre esses genes com a infecção pelo HPV.

Um estudo indiano encontrou maior frequência dos alelos nulos GSTM1 e GSTT1 em mulheres com câncer cervical e infectadas pelo vírus (JOSEPH et al., 2006). Sudenga et al. (2014) encontraram associação entre a infecção por HPV16 e a nulidade GSTM1 e GSTT1 e, entre, GSTM1 deletado com uma menor persistência (ou maior depuração/clearance) do subtipo de alto risco.

Em revisão sistemática, Bortolli et al. (2021) reuniram trabalhos que abordaram indiretamente a relação entre os SNPs, GSTM1 e GSTT1 e a infecção por HPV de alto risco oncogênico em mulheres com ou sem alterações cervicais

conforme (Tabela 1). E embora, ainda inconclusivo, o estudo apontou para uma frequência expressiva do alelo nulo GSTT1 em mulheres infectadas por subtipos de HPV de alto risco comparada ao GSTM1 deletado. Em função de lacunas sobre o assunto, investigações sobre a relação entre os SNPs e a infecção viral são necessárias, já que há indícios da associação deles com a progressão da infecção.

Tabela 1 - Resumo dos principais resultados, sobre a relação GSTM1 e GSTT1 e a infecção por HPV, encontrados na literatura.

Estudo	Polimorfismos genéticos	Resultados principais
Ueda et al., 2005	GSTM1, GSTT1 e p53 códon 72	Na população japonesa estudada, o alelo nulo GSTT1 foi mais frequente em mulheres com HSIL que apresentaram HPV de alto risco comparado as com LSIL, também com subtipos de alto risco e com controle. Nenhuma associação foi observada com GSTM1. Podendo o genótipo GSTT1 deletado estar relacionado com a progressão e severidade da lesão.
Ueda et al., 2010	GSTM1, GSTT1 e p53 códon 72	Os resultados sugeriram que o genótipo nulo GSTT1 pode aumentar em até 3,5 vezes o risco de HSIL e câncer do colo do útero, quando as mulheres estão infectadas por HPV de alto risco.
Lee et al., 2004	GSTM1, GSTT1 e p21	Os achados sugeriram um risco ampliado de 2,9 vezes para câncer do colo do útero em mulheres coreanas com GSTM1 nulo e com subtipos de alto risco. O GSTT1 nulo foi pouco frequente e apresentou relação de proteção ao câncer cervical. Contudo, possível interação entre os alelos GSTT1 nulo e p21 arg/arg ampliaram o risco em até duas vezes para o câncer nas infectadas.
Kim et al., 2000	GSTM1, GSTT1, p53, CYP1A1 e CYP2E1	Os resultados mostraram que mulheres com menos de 40 anos infectadas por HPV de alto risco tem maior frequência de GSTM1 nulo. E que o alelo deletado GSTT1 aumenta o risco para neoplasia cervical independentemente da idade. Assim como a dupla deleção simultânea, GSTM1 e GSTT1, amplia até 17,8 vezes a chance de desenvolvimento de carcinoma cervical nas mulheres com até 40 anos. Em contrapartida, os alelos nulos podem ser protetivos para aquelas com idade superior.
Cseh et al., 2011	GSTT1 e GSTM1	A presença dos genótipos nulos GSTM1 e GSTT1, esteve associado ao maior risco de desenvolver lesões pré-cancerosas em mulheres com infecções persistentes por subtipos de alto risco.
Sudenga et al., 2014	CYP1A1, GSTT1 e GSTM1	Os genótipos nulos GSTT1 e GSTM1 foram associados ao HPV 16. O alelo nulo GSTM1 também foi associado a maior taxa de depuração de HR-HPV. As variantes de GSTT1 não foi significativamente associado com tempo de eliminação do HR-HPV. Além disso, conclui que os genes do metabolismo de xenobióticos influenciam a história natural das infecções por HPV de alto risco e a progressão do câncer cervical.

Nunobiki et al., 2015	GSTM1, GSTT1 e NQO1	Os resultados reforçam que a ausência do alelo GSTT1 pode estar associado a lesões pré-cancerosas mais graves do colo uterino, especialmente nas infecções por HPV de alto risco. O polimorfismo GSTM1 não está associado nem a infecção e nem as lesões.

HISL: lesão intraepitelial escamosa de alto grau; HPV: papilomavírus humano; LSIL: lesão intraepitelial escamosa de baixo grau; HR-HPV: papilomavírus humano de alto risco.

2. OBJETIVOS

2.1 Geral

Determinar a prevalência do HPV e investigar a relação entre os polimorfismos, GSTT1 e GSTM1, com a infecção viral e fatores de risco associados em mulheres atendidas em unidades de saúde do município de Francisco Beltrão/PR.

2.2 Específicos

- Determinar a prevalência do HPV em grupo de mulheres.
- Identificar fatores associados a infecção viral e fatores de risco.
- Estimar a frequência alélica dos genes GSTM1 e GSTT1.
- Verificar a relação dos genótipos nulos com a infecção viral.

3. MATERIAIS E MÉTODOS

3.1 População de estudo

O estudo quantitativo, observacional e transversal incluiu 324 mulheres com idade entre 18 e 65 anos, que frequentam Unidades Básicas de Saúde (UBS) de Francisco Beltrão, para consulta ginecológica de rotina. Todas as participantes tiveram como critério de inclusão já terem tido a primeira relação sexual, sendo excluídas as mulheres na condição de gestante. O projeto foi aprovado pelo Comitê de Ética em Pesquisa com Seres Humanos (CEP) e Comissão Nacional de Ética em Pesquisa (CONEP) através do parecer nº 2.254.450 e CAAE: 72983817.5.0000.0107. Após receberem informações sobre a pesquisa, as mulheres que concordaram em participar assinaram o Termo de Consentimento Livre Esclarecido (TCLE) (Anexo I) para serem incluídas no estudo.

Para caracterizar a população e avaliar potenciais fatores associados à infecção pelo HPV, as mulheres foram entrevistas a partir de um questionário estruturado elaborado com base na literatura (NONNENMACHER et al., 2002; PARREIRA, 2009; OLIVEIRA et al., 2013) (Anexo II). Deste instrumento foram obtidas informações socioeconômicas (idade, escolaridade, renda, estado civil, raça), de comportamento sexual e ginecológico (idade da primeira relação sexual, número de parceiros sexuais na vida, paridade, método contraceptivo, passado de infecção ginecológica, resultado do último e atual exame Papanicolau) e hábitos de vida (consumo de tabaco, álcool, uso de preservativo e imunização).

As unidades de saúde onde ocorreram as coletas foram: Estratégia Saúde da Família (ESF) dos bairros Antônio de Paiva Cantelmo, Cristo Rei, Industrial, Pinheirinho e uma unidade de referência para atendimento em ginecologia e obstetrícia a nível ambulatorial, o Instituto da Mulher (IM) localizada no bairro Cango. A participante passou pela consulta de enfermagem na unidade de saúde, onde ocorreu a coleta de material para o exame ginecológico, Papanicolau. A escova endocervical utilizada na coleta foi acondicionada em tubo (tipo Falcon) com 2mL de soro fisiológico e mantidas a temperatura de -20ºC até a detecção viral (NONNENMACHER et al., 2002; DUARTE et al., 2017). A armazenagem do

material biológico, a extração do DNA, bem como realização da análise laboratorial via PCR foram conduzidas no Laboratório de Biologia Molecular e Genética da Universidade Estadual do Oeste do Paraná – UNIOESTE, do município de Francisco Beltrão. Os resultados da análise citológica foram fornecidos pelas UBSs, seguindo a classificação do Sistema Bethesda (2001) (SOLOMON et al., 2002; INCA, 2016).

3.2 Detecção do HPV

Uma alíquota de 200µl da amostra original foi utilizado para isolar o material genético total, seguindo o protocolo de extração e purificação "Biological Fluid/Blood Genomic DNA extraction kit" – "Purelink® Genomic DNA Mini Kit" (Invitrogen by Thermo Fisher Scientific, Carlsbad, California) conforme o fabricante e estocado em freezer a -20ºC. Para detecção molecular do HPV, foram utilizados iniciadores específicos para síntese in vitro da região codificadora do gene L1 do vírus, MY09 (5'-CGTCCMAARGGAWACTGATC-3') e MY11 (5'-GCMCAGGGWCATAAYAATGG-3'), obtendo um fragmento de 450 pb. O volume final de cada reação de PCR foi de 25 µl, contendo, 10 mM Tris–HCl, 2 mM de $MgCl_2$, 0,1 mM de dNTPs, 0,5 µM de cada *primer*, 1,25U de Taq DNA polimerase (Ludwig Biotecnologia Taq DNA polimerase, Brasil), acrescentando ao final 3,5µl do DNA total (50ng/µl). Para determinar a qualidade da extração, todas as amostras tiveram um segmento de 268pb do gene β-globina humano sintetizado, a partir dos primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) e PCO4 (5′-CAACTTCATCCACGTTCACC-3′) (TRUGILO et al., 2019; SAIKI et al., 1985). As amplificações de ambos os genomas foram processadas em termociclador Applied Biosystems Veriti Thermal Cycler (ThermoFisher Scientific, Germany), seguindo as etapas: 10min a 94ºC, seguida por 37 ciclos de 1 min a 94ºC; 1min a 55ºC e 1 min a 72ºC; finalizando com extensão por 10 min a 72ºC (SAIKI et al., 1985; JESUS et al., 2018, TRUGILO et al., 2019). Como controle positivo da detecção viral foi incluído uma amostra de DNA de células HeLLa, com genoma do HPV 16.

Todos os amplicons foram fracionados via eletroforese em gel de agarose 2%, corado com brometo de etídio, sob uma diferença de potencial de 150 volts por uma hora, visualizado sob luz ultra-violeta (UV) e fotodocumentados.

3.3 Genotipagem dos SNPs GSTM1 e GSTT1

Para verificar a susceptibilidade à infecção viral frente as variações polimorficas de GSTM1 e GSTT1, foi delineado um estudo caso-controle com 105 mulheres da população investigada. Os casos foram mulheres com presença do vírus e que foi possível caracterizar os alelos GST, ao todo 21. E os controles foram as mulheres sem a IST com a caracterização alélica, obtendo um número de 84 controles. Os casos e controles foram pareados por idade, ± 2 anos.

O método PCR Multiplex foi usado para determinar os polimorfismos genéticos GSTM1 e GSTT1. Os pares de iniciadores utilizados foram: Forward 5'-GAACTCCCTGAAAAGCTAAAGC-3' e reverse 5'-GTTGGGCTCAAATA TACGGTGG-3' e Forward 5'-TTCCTTACTGGTCCTCACATCTC-3' e Reverse 5'-TCACCGGATCATGGCCAGCA-3'. O primeiro produz um amplicon de 219pb e o segundo de 459pb, respectivos, aos genes GSTM1 e GSTT1 (KIRAN et al., 2010; PAN et al., 2014). As condições da PCR incluíram desnaturação inicial a 94ºC por 5mim, seguindo para 35 ciclos de 94ºC por 1 minuto, 58ºC por 1 minuto, 72°C por 1 minuto, finalizando a 72ºC por 10 minutos. Os genótipos foram determinados pela migração dos produtos em gel de agarose com adição de 2% de brometo de etídio. A visualização dos amplicons revelam a presença dos genes e a ausência, caracteriza os como nulos.

3.4 Análise estatística

Os dados foram tabulados e analisados no software Statistical Package for the Social Sciences (SPSS) versão 24.0 e Jasp v. 0.13. As variáveis continuas tiveram a distribuição normal verificada pelo teste de Kolmogorov-Smirnov ($p<0,05$) e assim como as demais foram categorizadas. O teste Qui-quadrado (X^2) de Pearson, de continuidade de Yates e teste exato de Fisher foram utilizados para comparações entre variáveis categóricas independentes e o desfecho da infecção viral, inclusive, para o estudo caso-controle. Como tamanho de efeito das análises

bivariadas, utilizou-se o V de Cramer, com as classificações: efeito fraco >0.05; efeito moderado >0.10; efeito forte >0.15; efeito muito forte >0.25 (FERGUSON, 2009).

As variáveis independentes selecionadas com valor de $p < 0,20$ nas análises bivariadas foram incluidas no modelo multivariado de regressão logística, método *stepwise*. Os resultados compreenderam as razões de chances ou odds ratios (OR), com erros estandardizados robustos e intervalos de confiança de 95%, correção de vício acelerado (BCa) em técnica de reamostragem bootstrap (1000 reamostras) (FRANCISCO et al., 2008). Para selecionar o melhor modelo explicativo da regressão logística, o teste de Hosmer and Lemeshow foi empregado (ponto de corte > 0.05), em conjunto com o Omnibus Test of Model Coefficients (ponto de corte < 0.05). Ainda, um menor valor do Critério de Informação de Akaike (AIC) e aumento da variância explicada (R^2 de Nagelkerke) foram considerados na escolha do modelo multivariado final.

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5. RESULTADOS

5.1 ARTIGO CIENTÍFICO 01

PREVALÊNCIA DO HPV E FATORES ASSOCIADOS EM UMA POPULAÇÃO DE MULHERES DO SUL DO BRASIL

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Resumo

O Papilomavírus Humano (HPV) causa a infecção sexualmente transmissível mais comum no mundo e está associado a vários tipos de câncer, sendo o cervical o quarto no ranking

de óbitos em mulheres. O estudo objetivou determinar a prevalência do HPV em um grupo de 324 mulheres do sistema público de saúde de Francisco Beltrão, sudoeste do Paraná, Brasil. Foram realizadas entrevistas para obter informações socioeconômicas, sexuais, ginecológicas e hábitos de vida. Após realizado exame Papanicolau, a escova endocervical foi utilizada para detecção viral através da Reação em Cadeia da Polimerase (PCR), com os iniciadores MY09 e MY11. A prevalência do HPV foi de 6,8%, sendo que destas 58,3% apresentavam alterações citopatológicas. Os principais achadas apontaram como fatores associados a infecção viral as alterações cervicais atuais, ampliando em 32 vezes a chance da presença do HPV, infecção vaginal recente e pertencer a raça distinta da branca.

Palavras-chave: Papilomavírus Humano, Infecção sexualmente transmissível, saúde da mulher, Teste Papanicolau, etnia.

1. Introdução

O Papilomavírus Humano (HPV) causa a infecção sexualmente transmissível (IST) mais comum no mundo e pode infectar tanto a pele quanto as mucosas, reunindo cerca de 220 subtipos distintos [1,2]. Destes, 40 subtipos têm potencial para infectar o trato ano-genital, dos quais 12 são definidos como de alto risco para o desenvolvimento do câncer cervical (CC), especialmente os subtipos 16 e 18 [3, 4, 5, 6]. Entretanto, o CC possui bom prognóstico se descoberto e tratado precocemente [7, 8].

Em termos globais, o câncer cervical ocupa a quarta posição na frequência dos tipos de cânceres mais diagnosticado e a quarta causa de morte por câncer em mulheres, com uma estimativa de 604.000 novos casos e 342.000 mortes em todo o mundo em 2020 [9]. No estado do Paraná, Brasil, o número de óbitos em 2019 foi de 336 mulheres [10].

A infecção pelo HPV ocorre através de microlesões na pele ou mucosas durante a relação sexual, favorecendo a entrada do vírus na camada basal das células no epitélio [11]. A maioria das infecções são eliminadas ou suprimidas por células mediadoras do sistema imunológico entre um e dois anos. Contudo, infecções persistentes estão fortemente ligadas ao desenvolvimento de lesões intraepiteliais escamosas pré-cancerosas, tipicamente classificadas como de baixo e alto grau de acordo com o Sistema Bethesda [12].

Embora a infecção pelo HPV seja o principal fator de risco para o CC, sozinho, é na maioria das vezes um evento insuficiente [12]. O vírus pode ser detectado em mulheres com citologia normal e nem todas as infectadas irão apresentar lesões no colo uterino e evoluir para o câncer [13, 14]. Logo, fatores como início precoce da atividade sexual [15, 16], a raça [17], uso de anticoncepcionais [18, 19], exposição ao tabaco [20, 17], fatores imunológicos e genéticos [21], coinfecção por mais de um subtipo viral [22, 23] e o estresse oxidativo [24] podem interferir na biologia viral e para progressão da doença.

De acordo com o estudo multicêntrico de AHMV [25], a prevalência do vírus no Brasil varia de 13,7 a 54,3%, dependendo da população e área geográfica entre outros fatores. O estudo recente de Colpani et al. [14], revelou prevalência média de 25,41% no território nacional. Estudo multicêntrico realizado entre 2016 e 2017 detectou a prevalência de 49,7% para infecção por HPV em mulheres e homens da faixa etária de 16 a 25 anos no Sul do país, o menor índice dentre as regiões avaliadas [17]. No mesmo estudo, ainda, foi determinado prevalência expressiva, variando de 10,4% e 24,5% entre mulheres sem qualquer alteração no colo uterino.

Diante da importância do tema para saúde pública e da mulher, o presente estudo teve como objetivo determinar a prevalência do HPV e investigar os fatores associados à infecção em mulheres do município de Francisco Beltrão, sudoeste do estado do Paraná. A expectativa com os resultados é traçar um perfil geral do grupo com a infeção viral, contribuindo para o delineamento de estratégias de prevenção à IST e, por conseguinte, minimizar a evolução patológica da infecção pelo HPV.

2. Materiais e métodos

2.1. População de estudo e comitê de ética (Ethics statement)

O estudo quantitativo e transversal incluiu 324 mulheres com idade entre 18 e 65 anos, que frequentam locais serviços públicos de saúde para consulta ginecológica de rotina do município de Francisco Beltrão, estado do Paraná, Brasil. Estes serviços incluíram Estratégia Saúde da Família (ESF) do bairro Antônio de Paiva Cantelmo, as Unidades Básicas de Saúde (UBS) localizadas nos bairros Cristo Rei, Industrial e Pinheirinho e uma unidade de referência para atendimento em ginecologia e obstetrícia a nível ambulatorial, o Instituto da Mulher (IM) localizada no bairro Cango. O principal critério de inclusão das mulheres no estudo foi terem tido a primeira relação sexual e aquelas na condição de gestante foram excluídas. O projeto foi aprovado pelo Comitê de Ética em Pesquisa com Seres Humanos (CEP) da Universidade Estadual do Paraná e Comissão Nacional de Ética em Pesquisa (CONEP) com parecer nº 2.254.450 e CAAE: 72983817.5.0000.0107. Após receberem informações sobre a pesquisa, as mulheres que concordaram em participar assinaram o Termo de Consentimento Livre Esclarecido (TCLE).

2.2 Procedimentos

Para caracterizar a população e avaliar potenciais fatores associados à infecção pelo HPV, as mulheres foram entrevistas a partir de um questionário estruturado elaborado com base na literatura [26, 27, 28]. Deste instrumento foram obtidas informações socioeconômicas (idade, escolaridade, estado civil, raça e renda), hábitos de vida (consumo de tabaco e bebida alcóolica) e de comportamento sexual e ginecológico (idade da primeira relação sexual, número de parceiros sexuais na vida, paridade, método contraceptivo, passado de infecção ginecológica

e vaginal recente, resultado do último e atual exame Papanicolau, imunização). Posteriormente, cada participante passou pela consulta ginecológica com médico especializado, onde ocorreu a coleta de material para o exame ginecológico Papanicolau. A escova endocervical utilizada no procedimento foi preservada e acondicionada em tubo (tipo Falcon) com 2mL de soro fisiológico e mantidas em freezer a -20ºC até análise molecular para detecção viral [26, 29]. A armazenagem do material biológico, a extração do material genético e detecção do HPV foram processadas no Laboratório de Biologia Molecular e Genética da Universidade Estadual do Oeste do Paraná, campus de Francisco Beltrão. Os resultados da análise citológica foram fornecidos pelos serviços públicos de saúde, seguindo a classificação do Sistema Bethesda (2001) [30, 31].

2.3 Análise molecular

Uma alíquota de 200µl da amostra original foi utilizada para isolar o material genético total das participantes, seguindo o protocolo de extração e purificação de ácidos nucleicos "Biological Fluid/Blood Genomic DNA extraction kit" – "Purelink® Genomic DNA Mini Kit" (Invitrogen by Thermo Fisher Scientific, Carlsbad, California) conforme instruções do fabricante e estocado e armazenado em freezer a -20ºC. Para detecção do HPV foi realizado a técnica da Reação em Cadeia da Polimerase (PCR). Os iniciadores específicos utilizados para síntese *in vitro* de um fragmento de 450pb da região codificadora do gene L1 do vírus foram MY09 (5'-CGTCCMAARGGAWACTGATC-3') e MY11 (5'-GCMCAGGGWCATAAYAATGG-3') [31]. O volume final de cada reação de PCR foi de 25 µl, contendo, 10 mM Tris–HCl, 2 mM de $MgCl_2$, 0,1 mM de dNTPs, 0,5 µM de cada *primer*, 1,25U de Taq DNA polimerase (Ludwig Biotecnologia Taq DNA polimerase, Brasil), acrescentando ao final 3,5 µl do DNA total (50ng/µl). Para determinar a qualidade da extração, todas as amostras tiveram um segmento de 268pb do gene β-globina humano sintetizado, a partir dos primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) e PCO4 (5′-CAACTTCATCCACGTTCACC-3′) [32, 33]. As amplificações de ambos os genomas foram processadas em termociclador Applied Biosystems Veriti Thermal Cycler (ThermoFisher Scientific, Germany), seguindo as etapas: 10min a 94ºC, seguida por 37 ciclos de 1 min a 94ºC; 1min a 55ºC e 1 min a 72ºC; finalizando com extensão por 10 min a 72ºC [33, 34, 32]. Como controle positivo da detecção viral foi incluído uma amostra de material genético conhecido, das células HeLLa, contendo genoma do HPV 16.

Todos os amplicons foram fracionados via eletroforese em gel de agarose 2%, corado com brometo de etídio, sob uma diferença de potencial de 150 volts por uma hora, visualizado sob luz ultra-violeta (UV) e fotodocumentados.

2.4 Análise estatística

Os dados foram processados nos softwares Statistical Package for the Social Sciences (SPSS) versão 24.0 e Jasp v. 0.13. As variáveis continuas tiveram a distribuição normal verificada pelo teste de Kolmogorov-Smirnov ($p<0,05$) e assim como as demais foram categorizadas. O teste Qui-quadrado (X^2), com correção de continuidade de Yates e teste exato de Fisher foram utilizados para comparações entre variáveis categóricas independentes e o desfecho da infecção viral. Como tamanho de efeito das análises bivariadas, utilizou-se o V de Cramer, com as classificações: efeito fraco >0.05; efeito moderado >0.10; efeito forte >0.15; efeito muito forte >0.25 [35].

As variáveis independentes selecionadas com valor de $p < 0,20$ nas análises bivariadas foram incluidas no modelo multivariado de regressão logística, método *stepwise*. Os resultados compreenderam as razões de chances ou odds ratios (OR), com erros estandardizados robustos e intervalos de confiança de 95%, correção de vício acelerado (BCa) em técnica de reamostragem bootstrap (1000 reamostras) [36]. Para selecionar o melhor modelo explicativo da regressão logística, o teste de Hosmer and Lemeshow foi empregado (ponto de corte > 0.05), em conjunto com o Omnibus Test of Model Coefficients (ponto de corte < 0.05). Ainda, um menor valor do Critério de Informação de Akaike (AIC) e aumento da variância explicada (R^2 de Nagelkerke) foram considerados na escolha do modelo multivariado final.

3. Resultados

As características sociodemográficas, ginecológicas, sexuais e relativas aos hábitos de vida das participantes estão apresentadas na Tabela 1. A prevalência da infecção pelo HPV foi de 6,8%, ou seja, em 22 das 324 mulheres foi detectado a presença do vírus. Os resultados mostraram, preliminarmente, que as variáveis raça e renda podem estar associadas ao desfecho da infecção e com efeito moderado (conforme V de Cramer, Tabela 1). O tabagismo e o consumo de bebida alcoólica também sugerem associações com a IST, contudo, apresentam

efeitos fracos. O número de parceiros sexuais na vida e de novos parceiros no último ano (p = 0,152 e p = 0,080, respectivamente) foram as variáveis de comportamento sexual incluídas nas análises, inclusive de efeito moderado para este último. Na mesma condição estão as variáveis ginecológicas, alterações cervicais atuais (p < 0.001), infecção vaginal recente (p = 0.029) e tempo de realização do último exame Papanicolau (p = 0.149). Dentre elas, a primeira foi a que apresentou o efeito mais forte para o desfecho da infecção viral (acima de 40%), as outras duas demonstraram efeito moderado (Tabela 1).

Na análise de regressão logística multivariada, o modelo selecionado explicou 24,2% do desfecho viral (p=0,047). Neste modelo, a presença de alterações cervicais atuais ampliam quase 33 vezes a chance da presença do HPV (OR_{adj}: 32,688; IC 95% 8,508-125,589; p<0,001). A presença de infecção vaginal recente aumentou em 2,7 vezes as chances de infecção por HPV (OR_{adj}: 2,773; IC 95% 1,048-7,341; p<0,040) e pertencer a outras raças que não a branca representou uma chance três vezes maior do desfecho viral (OR_{adj}: 3,058; IC 95% 1,056-8,857; p<0,039) (Tabela 2).

4. Discussão

O presente estudo encontrou uma prevalência de infecção pelo HPV de 6,8%. Ademais, os principais fatores associados à infecção incluíram presença de alterações cervicais atuais, infecção vaginal recente e raça.

Em comparação com prevalências reportadas em outros locais, a encontrada no presente estudo é inferior às relatadas para a mesma faixa etária na América do Sul, incluindo países como Chile, Paraguai, Bolívia e Brasil (18,1%) [6]. Já em nível regional, estudos realizados em outras cidades do Paraná mostraram dados divergentes. Por exemplo, uma prevalência de 2,2% foi reportada na cidade de Ubiratã [36], enquanto índices superiores foram encontrados em Maringá (33,8%) [37] e em Curitiba (48%) [25]. Ao comparar os dados com estudos realizados na região sul, sobretudo em municípios com características semelhantes a Francisco Beltrão, também foram encontradas variações quanto ao HPV. Em Cruz Alta estado do Rio Grande do Sul, a prevalência do HPV foi de 34% [38] e, posteriormente, em 15,7% das mulheres investigadas [1]. Em Rio Grande no mesmo estado, dados de 335 raspados cervicais indicaram que o HPV foi detectado em 24% das amostras [39]. Provavelmente, inúmeros fatores podem explicar essas variações, como os metodológicos, culturais e sociodemográficos. Nesse contexto, agregando o aspecto socioeconômico, o município de Francisco Beltrão, sudoeste

paranaense, é a sétima cidade com o melhor Índice de Desenvolvimento Humano (IDH) do estado (0,774), superior ao da capital (0,749) [40]. Embora a variável renda não tenha se mantido como fator associado a infecção viral que a maioria das mulheres que apresentaram o HPV (77,3%) são aquelas com limitações financeiras, beneficiadas por no máximo dois salários mínimos (Tabela 1). No geral, a pobreza é um determinante social associado com a vulnerabilidade das mulheres, podendo influenciar na adoção de medidas preventivas contra infecções sexualmente transmissíveis ou reduzir o acesso à informação e serviços de saúde [41, 42]. Todavia, no presente estudo, hipotetiza-se que a baixa prevalência da infecção por HPV (6,8%) pode refletir a estrutura e eficiência da rede de atenção à saúde oferecida pelo município, possibilitando a realização e o acompanhamento dos exames ginecológicos preventivos.

Ao todo, apenas 12 mulheres apresentaram alterações cervicais, e dessas o vírus foi detectado em sete (58,83%). Achado este que reflete as alterações cervicais como o principal fator associado a presença do HPV na população em estudo (OR: 32,688), uma vez que o vírus pode provocar modificações severas no epitélio do colo uterino [43, 44]. A Agência Internacional de Pesquisa em Câncer [45] afirma que cerca de 10 a 30% das mulheres com HPV detectável possuem anormalidades citológicas. Coser et al. [38] observaram anormalidades cervicais em 3% (10) das mulheres do seu estudo, contudo, destas apenas uma não estava infectada pelo vírus. Melo et al. [16] encontram o HPV em 49,7% das mulheres com alterações cervicais e 18,2% eram lesões de alto grau. Logo, os resultados do presente estudo reforçam que as alterações no colo uterino têm forte relação com a infecção por HPV. Nesse contexto, tem-se a instabilidade ocasionada por infecções paralelas que ampliaram em 2,7 vezes a chance da presença do vírus no grupo. A infecção vaginal, independente da origem, pode provocar inflamações e rompimento do epitélio cervical, favorecendo a invasão celular pelo HPV [46].

Estudos de Guidry [47] e Suehiro et al. [37] demonstraram que infecções não atuais, mas recentes, são potenciais fatores de risco tanto para infecções virais como a causada pelo HPV, quanto às alterações cervicais e carcinogênese. Estudo realizado com mulheres portadoras do vírus HIV mostrou associação entre a infecção por HPV e composição da microbiota vaginal [48]. A relação entre as alterações na microbiota vaginal e infecação por HPV e a ocorrência de vaginose bacteriana, especilamente por *Gardnerella*, provoca danos na mucosa e aumentam as chances de entrada do vírus [8, 49]. Embora lacunas sobre a relação entre a microbiota vaginal e a infecção por HPV ainda persistam, há promessas de estudos para avaliar intervenções na microbiota com intuito de reverter o curso da infecção viral [50]. E com isso propor medidas preventivas e de tratamento precoce antes que a infecção evolua.

Além das alterações cervicais e infecção vaginal, o modelo multivariado manteve a raça como fator preditivo da infecção pelo HPV. A população do estudo trata-se de mulheres predominantemente autodeclaradas como brancas, característica comum da região com descendência italiana, alemã e polonesa [51]. Contudo, 31,8% das que apresentaram o vírus pertencem a outras raças com risco até três vezes maior para a infecção viral. Diferenças raciais ou étnicas são observadas em taxas de incidência de câncer cervical invasivo, sendo até 29% maiores em mulheres negras, hispânicas e não hispânicas, em comparação com brancas não hispânicas [55]. Hariri et al. [52] demonstraram maior prevalência de HPV entre negros não hispânicos, seguidos por brancos hispânicos e não hispânicos. Um estudo realizado com mulheres de baixa renda nos Estados Unidos, mostraram que hispânicas nascidas no México tiveram menor frequência do HPV (16%), seguido de um aumento entre brancas não hispânicas (29%), hispânicas nascidos nos Estados Unidos (35%) e negras não hispânicas (39%) [53]. As explicações para as divergências na prevalência do HPV entre as raças e etnias não são claras, embora possam estar relacionadas as condições socioculturais [53, 56] e a fatores genéticos do hospedeiro [54].

Embora este estudo foi o primeiro a determinar a prevalência do HPV em mulheres atendidas em serviços de saúde em Francisco Beltrão, um município referência na área da saúde para outras localidades, de modo que ações preventivas e interventivas possam ser delineadas, algumas limitações da pesquisa devem ser levadas em consideração. Logo, citam-se possíveis lapsos de memória das participantes ao responderem os questionários, interferindo nos resultados. O número limitado de mulheres com a presença do vírus, embora seja um resultado positivo para a população investigada, pode ter negligenciado algumas variáveis reconhecidas como fator de risco à IST viral, gerando um possível viés estatístico. Logo, sugere-se ampliação da amostra em trabalhos futuros e condução de estudos de caráter longitudinal para acompanhamento da população e avaliação da persistência da infecção pelo HPV.

5. Conclusão

O estudo conduzido, de forma inédita no município de Francisco Beltrão, interior do Estado do Paraná revelou uma prevalência baixa do HPV entre mulheres atendidas pelo Sistema Público de Saúde. Em suma, indicou como fatores que podem estar ligados a infecção viral as alterações cervicais, a infecção vaginal recente e a raça das mulheres. Nesse sentido, cabe um alerta ao serviço de saúde e um olhar diferenciado às minorias raciais, cujo acesso ao

acompanhamento ginecológico de rotina e rastreamento regular do câncer cervical podem estar limitados.

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Tabela 1 – Características sociodemográficas, hábitos de vida, ginecológicas e sexuais com poder de efeito para a infecção por HPV de um grupo de mulheres do sudoeste do Paraná, Brasil.

Variáveis	Detecção do HPV		Efeito*	Valor de *p*
	Positiva (n*=22)	Negativa (n*=302)		
Socioeconômicas/demográficas				
Idade				
≤ 37 anos	10 (7,4%)	126 (92,6%)	0,019	0,732[a]
> 37 anos	12 (6,4%)	176 (93,0%)		
Escolaridade				
≤ 8 anos	10 (45,5%)	98 (32,5%)	0,069	0,212[b]
> 8 anos	12 (55,5%)	204 (67,5%)		
Estado civil				
Casada/união estável	13 (59,1%)	218 (72,2%)	0,073	0,286[b]
Solteira/divorciada/viúva	9 (40,9%)	84 (27,8%)		
Raça				
Branca	15 (68,2%)	254 (84,1%)	0,107	**0,104[b]**
Outras	7 (31,8%)	48 (15,9%)		
Renda				
≤ 2 salários mínimos**	17 (77,3%)	168 (56,0%)	0,108	**0,072[c]**
> 2 salários mínimos	5 (22,7%)	133 (44,0%)		
Hábitos de vida				
Tabagismo				
Sim e ex-fumante	8 (36,4%)	66 (21,9%)	0,087	**0,193[b]**
Não	14 (63,6%)	236 (78,1%)		
Consumo de bebida alcoólica				
Sim	14 (63,6%)	139 (46,0%)	0,089	**0,169[b]**
Não	8 (36,4)	163 (54,0%)		
Sexuais/ginecológicas				
Início da relação sexual				
≤ 18 anos	14 (63,6%)	212 (70,2%)	0,036	0,684[b]
> 18 anos	8 (36,4%)	90 (29,8%)		
N° de parceiros sexuais na vida				
Até dois parceiros	8 (36,4%)	165 (54,6%)	0,092	**0,151[b]**
Três ou mais	14 (63,6%)	137 (45,4%)		
N° novos parceiros no último ano				
Nenhum	14 (63,6%)	246 (81,5%)	0,113	**0,080[b]**
Pelo menos um	8 (36,4%)	56 (18,5%)		
Prática de sexo oral				
Sim	9 (40,9%)	119 (39,4%)	0,008	1,000[b]
Não	13 (59,1%)	183 (60,6%)		
Paridade				
Sem filhos	7 (31,8%)	59 (19,5%)	0,077	0,268[b]
Um ou mais	15 (68,2%)	243 (80,5%)		

Uso de anticoncepcional				
Usou	18 (81,8%)	237 (78,5%)	0,021	1,000[c]
Não usou	4 (18,2%)	65 (20,5%)		
Uso de preservativo				
Sim	5 (22,7%)	44 (14,6%)	0,057	0,350[c]
Não	17 (77,3%)	258 (85,4%)		
Histórico de IST				
Sim	3 (13,6%)	26 (8,6%)	0,044	0,431[c]
Não	19 (86,4%)	276 (91,4%)		
Alterações cervicais atuais				
Sim	7 (68,2%)	5 (1,7%)	0,402	**<0,001[c]**
Não	15 (31,8%)	297 (98,3%)		
Infecção vaginal recente				
Sim	12 (54,5%)	96 (31,8%)	0,121	**0,029[a]**
Não	10 (45,4%)	206 (68,2%)		
Resultado do Papanicolau anterior				
Normal	14 (63,6%)	202 (66,9%)	0,017	0,938[b]
Com alterações	8 (36,4%)	100 (33,1%)		
Tempo do último Papanicolau				
Até um ano	12 (54,5%)	184 (60,9%)	0,116	**0,149[c]**
Mais de dois anos	7 (31,9%)	106 (35,1%)		
Nunca fez	3 (13,6%)	12 (3,4%)		
Doses de vacina				
Nenhuma	19 (86,4%)	283 (93,7%)	0,073	**0,180[c]**
Pelo menos uma das doses	3 (13,6%)	19 (6,3%)		

Notas: ** (R$ 1.045,00);* valor do Efeito de V de Cramer; [a]Qui-quadrado de Pearson; [b]Qui-quadrado com correção de continuidade de Yates; [c]Teste Exato de Fisher. Em negrito, associações que seguiram para análise multivariada com p<0,20. HPV: Human Papillomavirus. IST: Infecção Sexualmente Transmissível.

Tabela 2 - Análise multivariada dos fatores associados a infecção pelo HPV no grupo de mulheres investigadas.

Variáveis	OR_{ajd} (IC 95%)	Valor de p
Alterações cervicais atuais		
Não	1 (Referência)	
Sim	32,688 (8,508 - 125,589)	< 0,001
Infecção vaginal recente		
Não	1 (Referência)	
Sim	2,773 (1,048 - 7,341)	0,040
Raça		
Branca	1 (Referência)	
Outras	3,058 (1,056 - 8,857)	0,039

Notas: OR_{ajd} = valor obtido da Regressão Logística multivariada e $R^2_{Nagelkerke}$= 0,242 ou 24,2%.

4.2 ARTIGO CIENTÍFICO 02

GSTT1 and GSTM1 polymorphisms with human Papillomavirus infection in women from southern Brazil: a case-control study

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Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abstract

Background Some important factors for the most common sexually transmitted infection (STI) in the world caused by human papillomavirus (HPV) are well defined as early sexual activity, use of contraceptives, tobacco smoking, immunological and genetic factors. This study aimed to investigate the relationship between polymorphisms, GSTM1 and GSTT1, and HPV infection and associated risk factors in a group of women assisted in the public health system in southwestern Paraná, Brazil. **Methods and results** A case-control study was designed, in which the case group consisted of 21 women with HPV, matched by age, with the control group, 84 women without the virus. Viral detection was conducted via Polymerase Chain Reaction (PCR) and GSTM1 and GSTT1 genotyping by Multiplex PCR. The results showed that the GSTT1 null allele was a protective factor against infection (OR_{adj}: 0.219; 95% CI 0.078-0.618; p=0.004). No relationship was observed for the GSTM1 gene. The smoking habit was defined as a risk factor (OR_{adj}: 3.678; 95% CI 1.111-12.171; p=0.033), increasing the chance for the presence of HPV by up to 3.6 times. **Conclusion** In short, this study showed in an unprecedented way, the relationship between GSTM1 and GSTT1 genetic polymorphisms and HPV. The relationship found portrays protection from viral infection and not from susceptibility in the female population of southern Brazil.

Keywords: Human papillomavirus; Genetic polymorphism; glutathione S-transferase; GSTM1; GSTT1; Tobacco.

Introduction

The Human Papillomavirus (HPV) is a virus capable of infecting both the skin and mucous membranes, comprising about 220 distinct subtypes [1]. From these, 40 have the potential to infect the anogenital tract, characterizing it as responsible for the most common sexually transmitted infection (STI) in the world [2]. Among the subtypes, 12 are defined as high risk for the development of cancer, including cervical (CC), with emphasis on 16 and 18 [3]. This type of cancer is the fourth most frequently diagnosed among women and the fourth leading cause of cancer death in the female population, with a global estimate of 604,000 new cases and 342,000 deaths in 2020 [4]. Although, HPV infection is the main risk factor for cervical cancer, alone, it is most often an insufficient event [5].

Several factors that play an important role in viral biology and disease progression are well defined in the literature, such as ethnicity [6], early onset of sexual activity [7], use of contraceptives [8], exposure to tobacco [6, 9], co-infection with more than one viral subtype [10], oxidative stress [11], immunological and genetic factors [12].

The cytosolic glutathione-S-tranferase (GST) belong to a family of enzymes involved in phase II of xenobiotic metabolism and are divided into eight gene classes: alfa (α), mu (μ), pi (π), theta (θ), sigma (σ), kappa (κ), omega (ω) and zeta (ζ) [13]. The GSTM1 and GSTT1 genes located on chromosome 1p13.3 and 22q11.23, respectively, exhibit insertion and deletion polymorphism, and the null allele characterizes the absence of enzymatic function [14]. This condition has been related to oxidative stress, which therefore may be associated with pathologies such as diabetes mellitus, prostate cancer [15], breast [16] and cervical cancer [17] and also, favor the replication of some pathogens such as the Acquired Immunodeficiency Virus (HIV) in different ethnic groups [18].

Although there are gaps, it is known that genes involved in the metabolism of xenobiotics can influence the natural history of HPV subtypes, especially those with high oncogenic risk [17]. Although several studies point to a relationship between the GSTM1 and GSTT1 null genotypes and cervical cancer [19] there are controversies among population groups, restricting the generalization of such association [20]. An Indian study found a higher frequency of GSTM1 and GSTT1 null alleles in women with cervical cancer infected with the virus [21]. However, most studies prioritize the relationship between deleted alleles with events triggered by viral infection and not with susceptibility to HPV infection [17, 22]. Sudenga et al. [17] found an association between HPV16 infection and

GSTM1 and GSTT1 nullity, and between GSTM1 deleted with a lower persistence (or higher clearance) of the high-risk subtype.

In the recent systematic review, conducted by the authors of the present research, were gathered works that indirectly addressed the relationship between SNPs, GSTM1 and GSTT1 and high-risk HPV infection in women with or without cervical alterations [23]. Although still inconclusive, the study pointed to an expressive frequency of the GSTT1 null allele in women infected with high-risk HPV subtypes compared to the deleted GSTM1. Due to inconsistencies on the subject and the absence, so far, of studies on the direct relationship between SNPs and viral infection, the present study aimed to investigate the relationship between polymorphisms, GSTM1 and GSTT1, with the infection caused by HPV and associated risk factors in a group of women assisted by the public health system in southwestern Paraná, Brazil.

Materials and Methods

Study population and sampling

A case-control study was conducted with 105 women who attended Health Units (HU) for routine gynecological consultation in the municipality of Francisco Beltrão, State of Paraná, Brazil, between September and October 2017. The HU included in the study were Family Health Strategy (FHS) Antônio de Paiva Cantelmo, Cristo Rei, Industrial, Pinheirinho and a reference in gynecology and obstetrics at an outpatient level, the Women's Institute (*Instituto da Mulher* - IM). All participants had the inclusion criteria of having had their first sexual intercourse, excluding women in the condition of pregnant women. The case and control groups were matched by age (± 2 years) comprising, respectively, 21 women with positive detection for HPV and 84 with no viral infection.

In addition to the GSTM1 and GSTT1 polymorphism, potential factors associated with HPV infection, such as tobacco smoking, alcohol consumption, use of contraceptives and vaginal infection, all with potential xenobiotic characteristics, were evaluated. In addition to race, a relevant variable when considering the investigated polymorphisms. All information on these variables were obtained through interviews conducted individually from a validated questionnaire [24].

During the gynecological consultation, the women underwent collection of material for the Pap smear, and the endocervical brush used in the collection was placed in a microtube with 2mL of saline solution, kept at a temperature of -20°C until virus detection [24]. The storage of biological material, extraction of genetic material, viral detection and GST polymorphisms were conducted at the Molecular Biology Laboratory of the *Universidade Estadual do Oeste do Paraná*, in Francisco Beltrão, Brazil.

The project was approved by the Research Ethics Committee involving Human Beings at the State University of western Paraná, report No. 2.254.450 and CAAE: 72983817.5.0000.0107. After receiving information about the research, the women who agreed to participate signed the Free and Informed Consent Form (FICF) (Appendix I) to be included in the study.

Total DNA extraction and HPV detection

A 200µl aliquot of the original sample was used to isolate the total genetic material, following the extraction and purification protocol "Biological Fluid/Blood Genomic DNA extraction kit" – "Purelink® Genomic DNA Mini Kit" (Invitrogen by Thermo Fisher Scientific, Carlsbad, California) according to the manufacturer's instructions and stored in a freezer at -20ºC. For molecular detection of HPV, specific primers were used for in vitro synthesis of the coding region of the virus L1 gene, MY09 (5'-CGTCCMAARGGAWACTGATC-3') and MY11 (5'-GCMCAGGGWCATAAYAATGG-3'), producing a fragment of 450 bp. The final volume of each PCR (Polymerase Chain Reaction) reaction was 25 µl, containing, 10 mM Tris–HCl, 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 µM each primer, 1,25U of Taq DNA polymerase (Ludwig Biotechnology Taq DNA polymerase, Brazil), adding 3.5µl of total DNA at the end (50ng/µl). To control the extraction and synthesis process, PCR of a 268bp segment of the human β-globin gene was performed in all samples, using primers GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PCO4 (5′-CAACTTCATCCACGTTCACC-3′) [25]. The amplifications of both genomes were processed in thermocycler Applied Biosystems Veriti Thermal Cycler (ThermoFisher Scientific, Germany), following the steps: 10min at 94ºC, followed by 37 cycles of 1 min at 94ºC; 1min at 55ºC and 1 min at 72ºC; finishing with extension for 10 min at 72ºC [25]. As a positive control for viral detection, a DNA sample from HeLLa cells (HPV 16) was included). All amplicons were fractionated via 2% agarose gel electrophoresis,

stained with ethidium bromide, visualized under ultraviolet light (UV) and photodocumented.

GSTM1 and GSTT1 genotyping

Genotyping was performed by Multiplex PCR. The primer pairs used were 5'-GAACTCCCTGAAAAGCTAAAGC-3' (forward) e 5'-GTTGGGCTCAAATA TACGGTGG-3' (reverse) for GSTM1 and 5'-TTCCTTACTGGTCCTCACATCTC-3' (Forward) and 5'-TCACCGGATCATGGCCAGCA-3' (reverse) for GSTT1. The first produces an amplicon of 219bp and the second of 459bp [26]. PCR conditions included initial denaturation at 94°C for 5min, followed by 35 cycles of 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, ending at 72°C for 10 minutes. The genotypes were fractionated via 2% agarose gel electrophoresis, stained with ethidium bromide, visualized and photodocumented. The presence of amplicons characterizes the functional alleles and the absence of null alleles.

Statistical analysis

The genotypic frequencies, GSTM1 and GSTT1, and the variables tobacco smoking, alcohol consumption, use of contraceptives, vaginal infection and race were determined and compared between cases and controls using the Chi-square test (X^2), with continuity correction for Yates and Fisher. Variables with a significance value less than 0.20 were used for multivariate logistic regression, with a 95% confidence interval and $p<0.05$, to determine the risk factors for STIs. All statistical analyzes were performed using the software Statistical Package for Social Sciences (SPSS) version 24.0.

Results

The genotypic pattern of the GSTM1 and GSTT1 genes can be seen in Figure 1 and the allele frequency distribution are shown in Table 1. The GSTM1 null allele (GSTM1-) was more frequent both in cases (57.1%) and in controls (75.0%). In contrast, the GSTT1 null (GSTT1-) was less frequent (38.1%) in the case group when compared to the GSTT1+

allele (61.9%) and was more frequent in the control group (73.8%). Furthermore, the most common allelic combination in women with HPV was GSTM1-/GSTT1+ and in the control group it was the double deletion.

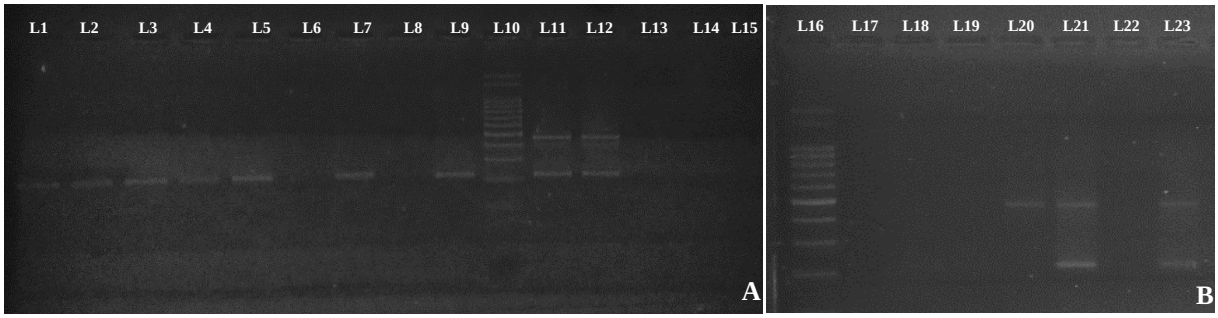

Fig. 1 Electrophoresis of PCR products from the co-amplification of GSTM1 and GSTT1. Ethidium bromide stained 2% agarose gel. Lanes (L): L1-L5, L7, L9 (Fig.1A) were positive for GSTM1; L6, L8, L13, L14 (Fig. 1A) and L18, L19 AND L22 (Fig.1B) were GSTM1 an GSTT1 deletion genotype. Lanes 11, L12, (Fig. 1A) and L21, L23 (Fig.1B) were positive for GSTM1 and GSTT1; L20 were positive for GSTM1 genotype (Fig.1B). Lanes 15 and 17 were negative control (Fig. A and B) and L10 and L16 were 100bp molecular weight marker (Fig. A and B).

Table 1 - Distribution of GST genotypes among the cases and controls of a group of women from the municipality of Francisco Beltrão, Paraná, Brazil.

Polymorphism	Cases (n=21)	Controls (n= 84)	Total (n= 105)
GSTM1			
Null/GSTM1-	12 (57.1%)	63 (75.0%)	75 (71.43%)
Present/ GSTM1+	9 (42.9%)	21 (25.0%)	30 (28.57%)
GSTT1			
Null/GSTT1-	8 (38.1%)	62 (73.8%)	70 (66.7%)
Present/GSTT1+	13 (61.9%)	22 (26.2%)	35 (33.3%)
Combinations			
GSTM1+/GSTT1+	6 (28.6%)	15 (17.9%)	21 (20%)
GSTM1+/GSTT1-	3 (14.3%)	7 (8.3%)	10 (9.53%)
GSTM1-/GSTT1+	7 (33.3%)	7 (8.3%)	14 (13.33%)
GSTM1-/GSTT1-	5 (23.8%)	55 (65%)	60 (57.14%)

Table 2 concentrates the results of the bivariate analysis (Chi-square test, X^2) suggesting associations between GST polymorphisms, life habits, vaginal infection, and race between groups. The choice of variables was based on the possible relationship with xenobiotic metabolism, which is directly linked to GST genes. All categories established for

the GST polymorphism suggested associations in the study (GSTM1, p=0.177; GSTT1, p=0.004; Combinations, p=0.002). As well as the smoking habit (p=0.040), the presence of recent vaginal infection (p=0.132) and race (p=0.043).

Although both the use of contraceptives and the consumption of alcohol did not show a significant association, 81% and 61.9% of women with HPV, respectively, use or have used the medication and consume some type of alcoholic beverage (Table 2).

Table 2 – Genetic polymorphism, life habits, vaginal infection and race associated with HPV in women from the municipality of Francisco Beltrão, Paraná, Brazil.

Variables	Cases (n= 21)	Controls (n= 84)	Total	*p* value
GSTM1				
Null/GSTM1-	12 (57.1%)	63 (75.0%)	75 (71.4%)	0.177[b]
Present/ GSTM1+	9 (42.9%)	21 (25.0%)	30 (28.6%)	
GSTT1				
Null/GSTT1-	8 (38.1%)	62 (73.8%)	70 (66.7%)	0.004[b]
Present/GSTT1+	13 (61.9%)	22 (26.2%)	35 (33.3%)	
Combinations				
GSTM1+/GSTT1+	6 (28.6%)	15 (17.9%)	21 (20.0%)	0.002[c]
GSTM1+ /GSTT1-	3 (14.3%)	7 (8.3%)	10 (9.5%)	
GSTM1-/GSTT1+	7 (33.3%)	7 (8.3%)	14 (13.3%)	
GSTM1 - /GSTT1-	5 (23.8%)	55 (65.5%)	60 (57.1%)	
Contraceptive				
No/Never used	4 (19.0%)	17 (20.2%)	21 (20.0%)	1.000[c]
yes and already used	17 (81.0%)	67 (79.8%)	84 (80.0%)	
Smoking habit				
Never smoked	14 (66.7%)	74 (88.1%)	88 (83.8%)	0.040[b]
Yes/ex-smoker	7 (33.3%)	10 (11.9%)	17 (16.2%)	
Alcohol consumption				
No	8 (38.1%)	42 (50.0%)	50 (47.6%)	0.464[b]
Yes	13 (61.9%)	42 (50.0%)	55 (52.4%)	
Vaginal Infection				
No	10 (47.6%)	55 (65.5%)	65 (61.9%)	0.132[a]
Yes	11 (52.4%)	29 (34.5%)	40 (38.1%)	
Race				
White	14 (66.7%)	72 (85.7%)	86 (81.9%)	0.043[b]

Others	7 (33.3%)	12 (14.3%)	19 (18.1%)

Notes: [a]Pearson's chi-square test; [b]Chi-square with Yates's correction for continuity; [c]Fisher's exact test. Associations that followed for multivariate analysis with p<0.20.

Table 3 shows the variables that remained in the multivariate analysis and defined an explanatory model for viral infection. The GSTT1 null allele was defined as a protective factor (OR_{adj}: 0.219; 95% CI 0.078-0.618; p<0.004). In other words, women who have GSTT1 deletion are less susceptible to HPV infection than the control group. On the other hand, smoking habit was recognized as a risk factor, increasing the chance of viral infection up to 3.6 times (OR_{adj}: 3.678; 95% CI 1.111-12.171; p<0.033). No interaction was observed between variables.

Table 3 – Predictive factors for HPV infection in a group of women living in the municipality of Francisco Beltrão, Paraná, Brazil, based on multivariate logistic regression analysis.

Variables	OR_{adj}	95% CI	*p* value
GSTM1			
Null	0.960	(0.289 - 3.189)	0.947
Present	1 (Reference)		
GSTT1			
Null	0.219	(0.078 - 0.618)	0.004
Present	1 (Reference)		
Smoking habit			
Yes/ex-smoker	3.678	(1.111- 12.171)	0.033
Never smoked	1 (Reference)		
Recent Vaginal Infection			
Yes	2.140	(0.745 - 6.145)	0.158
No	1 (Reference)		
Race			
Others	1.825	(0.537- 6.208)	0.335
White	1 (Reference)		

Notes: OR_{ajd} = value obtained from the Multivariate Logistic Regression with p<0.05 and $R^2_{Nagelkerke}$= 0.20 or 20%.

Discussion

As far as we know, the results of the present study have shown, in an unprecedented way, that the GSTT1 null allele was characterized as a protective factor against viral

infection, that is, women with GSTT1 deletion are less susceptible to HPV infection than the control group. While smoking habit was defined as a risk factor, increasing the chance of viral infection up to 3.6 times.

In general, individuals with GSTM1 and GSTT1 null genotypes are mainly predisposed to damage caused by oxidative stress [27]. Although there are gaps, it is known that genes involved in the metabolism of xenobiotics can influence the natural history of HPV subtypes, especially those with high oncogenic risk [17]. Individuals with GSTM1 and GSTT1 null alleles do not conjugate metabolics during phase II of xenobiotic metabolism, increasing damage caused by oxidative stress, facilitating infection and viral replication [13].

The GSTM1 and GSTT1 SNPs have been reported in various pathologies such as rheumatoid arthritis, age macular degeneration, prostate cancer, lung cancer, cervical cancer, schizophrenia [28, 29, 30]. In addition, a recent study suggests that patients with severe acute respiratory syndrome caused by Sars-cov-2 (COVID-19) with GSTT1 null genotype had higher mortality compared to those with the presence of the active allele [31]. Although several studies point to a relationship between the deletions, GSTM1 and GSTT1, and risk for cervical cancer [19, 32], there is still controversy among population groups, restricting the generalization of such association [20]. In this context, it is reiterated that the vast majority of studies prioritize the relationship between the deleted alleles with the course and not with the susceptibility of the infection [22, 17].

Several studies suggest that individuals with homozygous deletion of both genes have limitation or incapacity in the production of their enzymes, decreasing the ability of the detoxification process and increasing the malignant transformation of cells promoting carcinogenesis [33]. Among the cancers related to the presence of the virus are head and neck [34] and cervical cancer [19].

The findings of the present study showed no relationship between the GSTM1 polymorphism and viral infection. However, it showed for the first time that the deleted GSTT1 decreases the chances of HPV infection, offering protection to women with the null genotype (GSTT1-). Research conducted in patients with HIV suggests that deletions in the GSTM1 and GSTT1 genes lead to slower disease progression [18]. Iorio et al. [35] reinforce that GSTT1- is a protective genetic marker for allergic rhinitis. In other work, both the GSTT1 deleted allele and the combined form with the GSTM1 null are protective factors and reduced the susceptibility to schizophrenia in the Chinese population [29].

Chen e Nirunsuksir [36] observed that HPV infection behaves differently according to GST genotypes. The product of GSTT1 is believed to protect the host's DNA against

mutations [37]. Foppoli et al. [38] reported that high-risk subtypes are able to modulate and counteract the effects of increased levels of ROS (reactive oxygen species) in infected cells. That is, viral oncoproteins allow infected cells to survive in a hostile oxidizing environment, preventing the oxidation of anti-apoptotic and detoxifying enzymes. E6 and E7 modulate cellular microRNAs that regulate genes associated with the antioxidant response, suppression of OS-induced apoptosis and regulation of antioxidant enzymes and compounds [39].

The activity of several antioxidant proteins, such as those from the catalases family, peroxiredoxins, quinone oxidoreductase-1 and superoxide dismutase (SOD), can be disrupted by viral infections [39]. Furthermore, the expression of oncoproteins is associated with high levels of detoxifying enzymes, such as GSTs and GSH, providing the host cell with an escape system from oxidative damage and resisting programmed cell death [39].

One of the only works with results similar to those presented was that by Lee et al. [22], who found a protective relationship of the GSTT1 null allele in women infected with high-risk HPV, even with the development of cervical cancer. However, they did not evaluate the relationship of susceptibility to infection. A recent systematic review suggested a risk association between null GSTT1 and infection by high-risk HPV subtypes [23]. Numerous works reiterate the probable influence of GSTM1 and GSTT1 null alleles for the progression of several types of cancer, including cervical cancer [19, 28]. However, this finding is still not unanimous. The approach of these polymorphisms with the infection by HPV and other viral agents are scarce, however, they are fundamental for the understanding of results like the ones presented.

Unlike the GST polymorphism, smoking habit was identified as a risk factor for viral infection. In the present study, the risk of HPV infection in smokers and ex-smokers is 3.6 higher compared to those who never smoked. A Finnish study revealed a 1.76-fold higher susceptibility to infection, especially of subtype 16, in female smokers [40]. Another study, conducted in Greece, showed that women who smoke are 1.7 times more likely to be infected [41]. Several studies bring tobacco smoking as a risk factor that increases the risk of the presence of HPV in different populations [42]. Tobacco smoking affects the immune system, increases the risk of new virus infections, as well as the persistence of HPV [43]. Simen-Kapeu et al. [43] reported that young female smokers have limitations in the production of antibodies to fight high-risk oncogenic subtypes. A similar finding was revealed in a study by Bergqvist et al. [40]. However, the influence of smoking in the initial phase of HPV infections is not yet fully understood as to cervical carcinogenesis [44]. Tobacco metabolites

present in uterine cervical mucus decrease the quantity and function of epithelial Langerhans cells, producing a local immunosuppressive effect, leading to the host's inability to develop an effective immune response, maximizing the risk of viral and persistent infections, such as those of HPV [45, 46]. Tobacco consumption can also exacerbate the carcinogenic action of the virus by inhibiting INF-γ, TNF-α and mutations in the p53 gene, preventing apoptosis of the infected cell, favoring the progression of infection to cervical lesions and cancer [47]. Furthermore, some components of cigarettes such as benzopyrene, nicotine and derivatives damage the cervical epithelium, favoring viral infection [47].

In this work, it can be inferred that the smoking habit characterized the exposure to xenobiotics in the female population and proved to be a predictive factor for the risk of viral infection. Tian et al. [33] suggested a relationship between GSTT1 deletion and the chance of cervical changes in smokers. However, in the findings of this and other studies [19], no interaction was observed between GST polymorphisms and smoking in the investigated population. Still, the study has some limitations. The small number of women with HPV, interfering with the statistical power of the analyses. Condition that can be solved by expanding the sample and including other populations to validate the results presented. However, the case-control study also showed strengths points and allowed us to conclude that tobacco smoking is an important risk factor for viral infection. Furthermore, it was the first work to effectively assess the relationship between GSTM1 and GSTT1 and STI caused by HPV, demonstrating that the GSTT1 null allele can protect women from infection.

Conclusion

The study conducted in the municipality of Francisco Beltrão, in the State of Paraná, revealed unprecedented results and characterized the GSTT1 null allele as a protective factor against viral infection, that is, women who have GSTT1 deletion are less susceptible to HPV infection than the control group. While smoking habit was defined as a risk factor, raising up to 3.6 times the chance for viral infection.

In this sense, it is worth other studies that can expand the sample, allowing dividing the groups between GSTT1-/GSTT1+ genotypes and smokers and non-smokers, and see if there is a relationship between them.

Acknowledgements

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The *Universidade Estadual do Oeste do Paraná* that guaranteed access to the Molecular Biology laboratory where the samples were processed.

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Compliance with ethical standards

Funding

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Conflict of interest

All authors declare that they have no conflict to interest.

Author Contribution

Conceptualization: Ana Paula Reolon Bortolli and Léia Carolina Lucio; **Methodology:** Ana Paula Reolon Bortolli, Valquíria Kulig Vieira, Indianara Carlotto Treco, Claudicéia Risso Pascotto, Guilherme Welter Wendt, and Léia Carolina Lucio; **Formal analysis and investigation:** Ana Paula Reolon Bortolli, Guilherme Welter Wendt, and Léia Carolina Lucio; **Writing - original draft preparation**: Ana Paula Reolon Bortolli and Léia Carolina Lucio; **Writing - review and editing:** Ana Paula Reolon Bortolli, Valquíria Kulig Vieira, and Léia Carolina Lucio; **Resources:** Indianara Carlotto Treco and Léia Carolina Lucio; **Supervision:** Ana Paula Reolon Bortolli and Léia Carolina Lucio.

Consent to Participate

All authors informed consent.

Consent to Publish

All authors approved the version to be published and agree to be accountable for all aspects of the work in ensuring related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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ANEXO I

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - TCLE

Título do Projeto: PERFIL EPIDEMIOLOGICO E IDENTIFICAÇÃO DO PAPILOMAVÍRUS HUMANO (HPV) POR BIOLOGIA MOLECULAR EM MULHERES

(Indianara C. Treco – 46999764010; Léia Carolina Lucio – 46999332938)

Convidamos **a Sra. ou Senhorita** a participar de nossa pesquisa que tem o objetivo de **Identificar os fatores associados à infecção por HPV e os genótipos mais frequentes em mulheres atendidas pelo Sistema Único de Saúde, no Munícipio de Francisco Beltrão- PR, Brasil.** Esperamos, com este estudo, **possibilitar a adoção de intervenções e medidas estratégicas para fortalecimento e redirecionamento das políticas e programas regionais de controle do câncer do colo do útero.** Para tanto, solicitamos a sua participação respondendo o presente questionário e autorizando o uso do descarte do exame citopatológico, garantimos o sigilo quanto a sua identificação na pesquisa. Durante a execução do projeto os possíveis riscos estão relacionados a algum constrangimento quanto algumas perguntas presentes no questionário durante a entrevista. Caso ocorra o constrangimento, o pesquisador tranquilizará o entrevistado informando que o objetivo da pesquisa é científico e que tem obrigação de resguardar o entrevistado e o sigilo sobre sua identidade. Sua identidade não será divulgada e seus dados serão tratados de maneira sigilosa, sendo utilizados apenas fins científicos. Você também não pagará nem receberá para participar do estudo. Além disso, você poderá cancelar sua participação na pesquisa a qualquer momento. No caso de dúvidas ou da necessidade de relatar algum acontecimento, você pode contatar os pesquisadores pelos telefones mencionados acima ou o Comitê de Ética pelo número 3220-3092. Este documento será assinado em duas vias, sendo uma delas entregue ao sujeito da pesquisa.

Declaro estar ciente do exposto e **(desejo participar do projeto)** ou **(autorizo nome do menor)** a participar da pesquisa.

_____

(Assinatura da entrevista ou responsável)

Eu, **(Léia Carolina Lucio ou Indianara Carlotto Treco)**, declaro que forneci todas as informações do projeto ao participante e/ou responsável.

Indianara Carlotto Treco

Indianara Carlotto Treco (responsável 1)

Léia Carolina Lucio

Léia Carolina Lucio (responsável 2)

ANEXO II

INSTRUMENTO DE COLETA DE DADOS

INICIAIS NOME: _____ **Nº REQUISIÇÃO**:_____

1) NASCIMENTO: ___/___/___ **IDADE** _____/--

2) RAÇA:_____

1.Branca; 2.Negra; 3.Parda; 4.Indígena; 5.Amarela.

3) ESTADO CIVIL: _____

1.Casada; 2.Solteira; 3. Divorciada, viúva. 4. União estável

4) PARIEDADE: _____

1.Nenhum; 2.Um; 3.Dois; 4. Mais que três.

5) ESCOLARIDADE: _____

1.Nenhuma; 2.Ensino Fundamental incompleto; 3. Ensino Fundamental Completo; 4. Ensino Médio incompleto; 5. Ensino Médio Completo; 6. Ensino Superior incompleto; 8. Ensino Superior completo.

6) RENDA:_____

7) NÚMERO DE PARCEIROS SEXUAIS NA VIDA:_____

1.Um; 2.Dois; 3.Três; 4.Mais que quatro.

8) IDADE DA PRIMEIRA RELAÇÃO SEXUAL:_____

1. ≤14 anos; 2.15-16 anos; 3.17-18anos; 4.19-21 anos; 5. ≥22 anos.

9) USA ANTICONCEPCIONAL_____

1.Sim; 2.Não; 3.Nunca usou; 4.Não mais.

10) TEMPO DE USO_____

1. < 1 ano; 2. 1-5 anos; 3.> 5 anos;

11) USA PRESERVATIVO NA RELAÇÃO SEXUAL ____

1. Sim; 2. Não.

12) HISTÓRIA DE DST _____

1. sim; 2.não.

13) CASO A RESPOSTA ANTERIOR FOR: SIM.

QUAL?

_____

14) OCORRÊNCIA RECENTE DE INFECÇÃO VAGINAL____

1. sim; 2.não.

15) ÚLTIMO EXAME GINECOLÓGICO (EXAME DO PAPANICOLAU) ____

1. ≤ 1 ano; 2. ≥ 2 anos.

16) RESULTADO DO ÚLTIMO EXAME GINECOLÓGICO (EXAME DO PAPANICOLAU) ____

1. Normal; 2. Alterado.

17) FUMA ____

1. sim; 2. Não; 3 ex. fumante.

ANEXO III

NORMAS REVISTA DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

Disponível em: https://www.elsevier.com/journals/diagnostic-microbiology-and-infectious-disease/0732-8893/guide-for-authors

AUTHOR INFORMATION PACK TABLE OF CONTENTS

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Please ensure the figures and the tables are placed at the end of the manuscript, after the references. Tables and may be submitted as separate files, or may be included at the end of the manuscript document. The corresponding captions should be included for each figure or table.

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Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

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Results should be clear and concise.

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This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq.

(A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

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• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are optional yet highly encouraged for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

A concise and factual abstract is required. It should be 150 words or less for full-length papers and 50 words or less for notes. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp.

Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Indicate per figure if it is a single, 1.5 or 2-column fitting image.

• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.

• Please note that individual figure files larger than 10 MB must be provided in separate source files.

A detailed guide on electronic artwork is available.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.

TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.

TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.

• Supply files that are too low in resolution.

• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

Illustration services

Elsevier's Author Services offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce scientific, technical and medical-style images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables
Please submit tables as editable text and not as images. Tables can be placed either

next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is highly encouraged.

A DOI is guaranteed never to change, so you can use it as a permanent link to any electronic article. An example of a citation using DOI for an article not yet in an issue is: VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, https://doi.org/10.1029/2001JB000884. Please note the format of such citations should be in the same style as all other references in the paper.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author

name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

Users of Mendeley Desktop can easily install the reference style for this journal by clicking the following link:

http://open.mendeley.com/use-citation-style/diagnostic-microbiology-and-infectious-disease
When preparing your manuscript, you will then be able to select this style using the Mendeley plug-ins for Microsoft Word or LibreOffice.

Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;

2. *Two authors:* both authors' names and the year of publication;

3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.
Citations may be made directly (or parenthetically). Groups of references can be listed either first alphabetically, then chronologically, or vice versa. Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999)….

Or, as demonstrated (Jones, 1999; Allan, 2000)… Kramer et al. (2010) have recently

shown …'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:
Reference to a journal publication:

Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. J Sci Commun 2010;163:51–9. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

Van der Geer J, Hanraads JAJ, Lupton RA. The art of writing a scientific article. Heliyon. 2018;19:e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.

Reference to a book:

Strunk Jr W, White EB. The elements of style. 4th ed. New York: Longman; 2000.
Reference to a chapter in an edited book:

Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age. New York: E-Publishing Inc; 2009. p. 281–304.

Reference to a website:

Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).

Reference to a dataset:

[dataset] Oguro M, Imahiro S, Saito S, Nakashizuka T. Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1; 2015. https://doi.org/10.17632/xwj98nb39r.1.

Reference to software:

Coon E, Berndt M, Jan A, Svyatsky D, Atchley A, Kikinzon E, et al. Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88). Zenodo; 2020 (March 25). https://doi.org/10.5281/zenodo.3727209.

Note shortened form for last page number. e.g., 51–9, and that for more than 6 authors the first 6 should be listed followed by "et al." For further details you are referred to "Uniform Requirements for Manuscripts submitted to Biomedical Journals" (J Am Med Assoc 1997;277:927–34) (see also Samples of Formatted References).

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations.

Video

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the file in one of our recommended file formats with a preferred maximum size of 150 MB per file, 1 GB in total. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

ANEXO IV

NORMAS REVISTA MOLECULAR BIOLOGY REPORTS

Disponível em: https://www.springer.com/journal/11033/submission-guidelines

Original Research Article Guidelines

1. Maximum Word Count: 5,000 words

2. References: 50 or less

3. Results and Discussion: sections should be separate, not combined,

4. Figures and/or Tables: maximum 5 separate figures/tables. A figure should have a maximum of 4 panels.

Abstract Format

Max Word Count: 250 words

Format: The abstract should be presented divided into subheadings as follows (unless it is a mini or full review article):

I. Background: Brief summary of basic, relevant background info (2-3 sentences). Rationale and

purpose of the study.

II. Methods and Results: Brief explanation of experimental procedure and presentation of

significant results. Include sample sizes as well as animal species if applicable.

III. Conclusions: Succinct interpretation of results as well as significance of findings. Statement

of the main conclusion of the study. Emphasis should be on new information found during the study.

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink “Submit manuscript” on the right and upload all of your manuscript files following the instructions given on the screen.

Please ensure you provide all relevant editable source files. Failing to submit these source files might cause unnecessary delays in the review and production process.

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

- The name(s) of the author(s)
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

- Trial registration number and date of registration for prospectively registered trials
- Trial registration number and date of registration, followed by “retrospectively registered”, for retrospectively registered trials

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Statements and Declarations

The following statements should be included under the heading "Statements and Declarations" for inclusion in the published paper. Please note that submissions that do not include relevant declarations will be returned as incomplete.

- **Competing Interests:** Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Please refer to “Competing Interests and Funding” below for more information on how to complete this section.

Please see the relevant sections in the submission guidelines for further information as well as various examples of wording. Please revise/customize the sample statements according to your own needs.

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature’s LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The entries in the list should be numbered consecutively.

If available, please always include DOIs as full DOI links in your reference list (e.g. “https://doi.org/abc”).

- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

If you are unsure, please use the full journal title.

Authors preparing their manuscript in LaTeX can use the bibliography style file sn-basic.bst which is included in the Springer Nature Article Template.

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

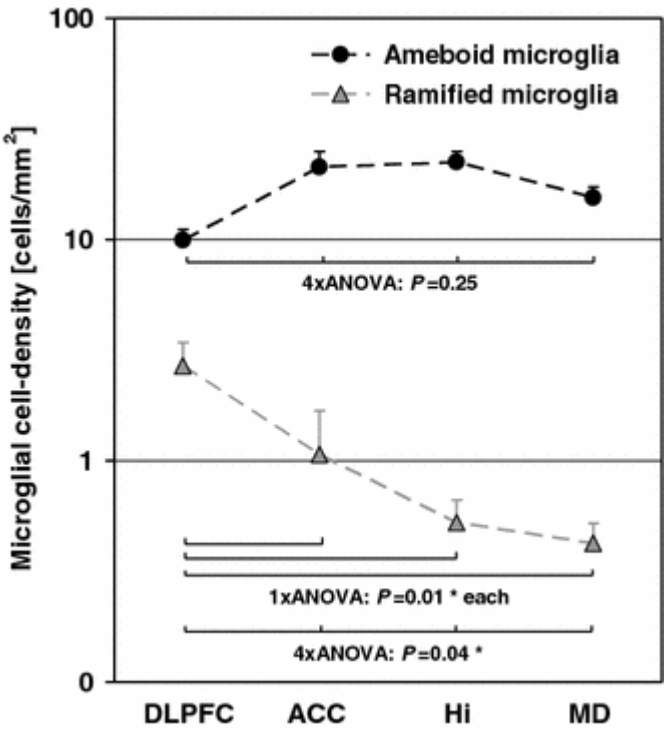
Artwork and Illustrations Guidelines

Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.

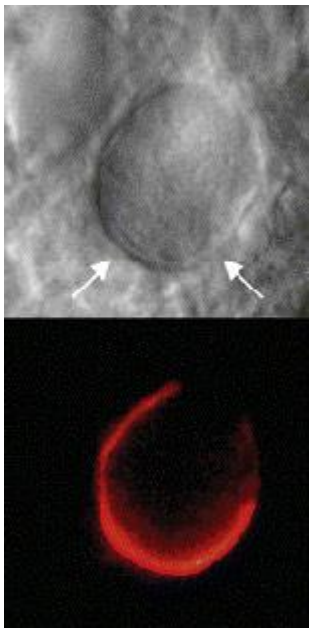
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



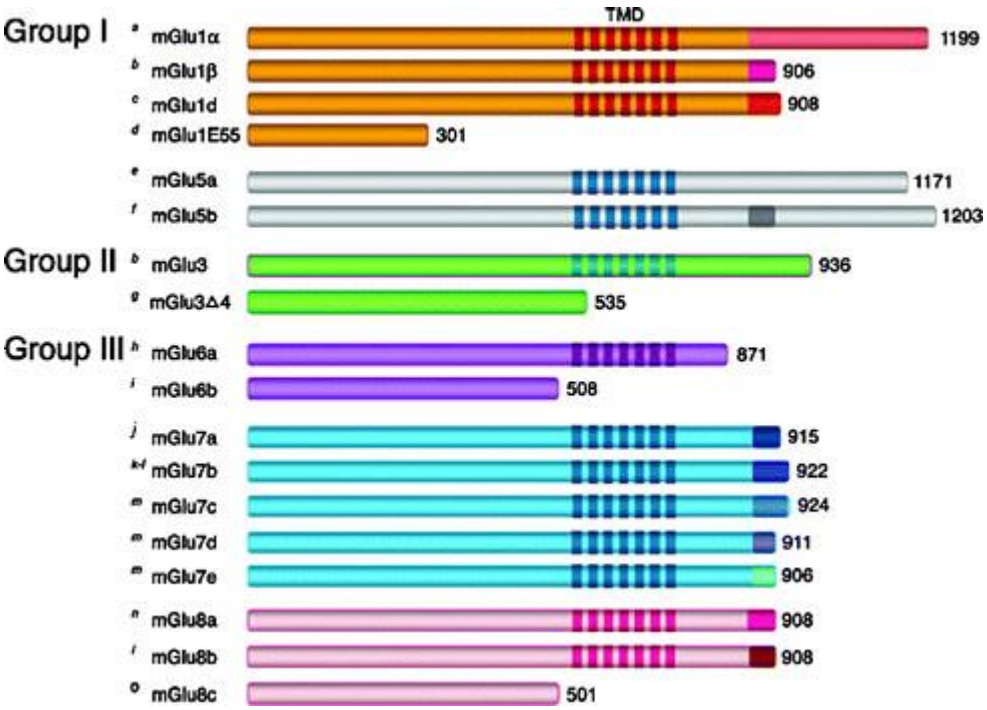
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,"A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.

- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

- Figures should be submitted separately from the text, if possible.
- When preparing your figures, size figures to fit in the column width.
- For large-sized journals the figures should be 84 mm (for double-column text areas), or 174 mm (for single-column text areas) wide and not higher than 234 mm.
- For small-sized journals, the figures should be 119 mm wide and not higher than 195 mm.

Permissions

If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)
- Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements)
- Any figure lettering has a contrast ratio of at least 4.5:1

Supplementary Information (SI)

Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

Before submitting research datasets as Supplementary Information, authors should read the journal’s Research data policy. We encourage research data to be archived in data repositories wherever possible.

Submission

- Supply all supplementary material in standard file formats.

- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.
- High resolution (streamable quality) videos can be submitted up to a maximum of 25GB; low resolution videos should not be larger than 5GB.

Audio, Video, and Animations

- Aspect ratio: 16:9 or 4:3
- Maximum file size: 25 GB for high resolution files; 5 GB for low resolution files
- Minimum video duration: 1 sec
- Supported file formats: avi, wmv, mp4, mov, m2p, mp2, mpg, mpeg, flv, mxf, mts, m4v, 3gp

Text and Presentations

- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

Specialized Formats

- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
- Refer to the supplementary files as “Online Resource”, e.g., "... as shown in the animation (Online Resource 3)", “... additional data are given in Online Resource 4”.
- Name the files consecutively, e.g. “ESM_3.mpg”, “ESM_4.pdf”.

Captions

- For each supplementary material, please supply a concise caption describing the content of the file.

Processing of supplementary files

- Supplementary Information (SI) will be published as received from the author without any conversion, editing, or reformatting.

Accessibility

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

- The manuscript contains a descriptive caption for each supplementary material
- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

ANEXO V

Comprovante de submissão Artigo 02, dia 08/11/2021