Contributo para a melhoria do rastreio do cancro do colo do útero em Portugal

Vitor Manuel Branco e Silva Caeiro

Tese para obtenção do Grau de Doutor em
Medicina
(3º ciclo de estudos)

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27 de fevereiro de 2026

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Dedicatória

À memória da minha mulher, cuja coragem, resiliência e força diante da adversidade, foram uma inspiração para todos os que tiveram o privilégio de com ela conviver.

A sua luta contra o cancro faz-nos ter presente a importância da pesquisa e da consciencialização sobre esta doença.

Que a sua história e legado continuem a iluminar o caminho na prossecução da cura e da esperança.

A morte chega cedo,
Pois breve é toda a vida
O instante é o arremedo
De uma coisa perdida.

O amor foi começado,
O ideal não acabou,
E quem tenha alcançado
Não sabe o que alcançou.

E tudo isto a morte
Risca por não estar certo
No caderno da sorte
Que Deus deixou aberto.

Fernando Pessoa, in "Cancioneiro"

Agradecimentos

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A todos os que de qualquer forma contribuíram para tornar possível este projeto.

Resumo

O cancro constitui, neste século, um dos principais problemas de saúde pública, com implicações sociais e económicas significativas. Globalmente, é responsável por aproximadamente 16,8% de todas as mortes e por 22,8% das mortes atribuídas a doenças não transmissíveis. O cancro do colo do útero (CCU) é um dos problemas de saúde pública mais significativos para a população feminina em todo o mundo, sendo o quarto cancro mais comum em termos de incidência e mortalidade nas mulheres, com uma estimativa de 662.301 novos casos e 342.000 mortes em todo o mundo em 2022, segundo a World Health Organization (WHO) 2023. Caracterizado pela sua elevada incidência e mortalidade, particularmente em países de Índice de Desenvolvimento Humano (IDH) baixo ou médio, este tipo de cancro está maioritariamente associado à infeção persistente pelo vírus do papiloma humano (HPV), e estima-se que mais de 90% dos casos de CCU sejam atribuídos aos genótipos de alto risco deste agente viral (HPV-hr).

O CCU é um problema de saúde significativo também em Portugal, onde em 2020, foi determinada uma incidência e mortalidade padronizada por idades de 10,7 e 3,2/100 000 mulheres-ano, respetivamente. De acordo com os dados do The Global Cancer Observatory (IARC/WHO) 2024, a Incidência e Mortalidade em Portugal por CCU em 2022, foram de 897 e 459, respetivamente, tendo sido nesse ano, o 8.º cancro mais frequente entre as mulheres e o 3.º cancro mais frequente entre as mulheres entre os 15 e os 44 anos. A taxa de mortalidade por CCU em Portugal, situa-se nos níveis muitos altos de IDH.

A WHO recomenda a implementação de programas de rastreio organizados, baseados na população, como a estratégia mais eficaz para a prevenção do CCU. No entanto, o rastreio oportunístico continua a desempenhar um papel importante na prevenção secundária do CCU em muitos países, incluindo Portugal, complementando os esforços do programa de rastreio, colmatando as dificuldades de acesso ao rastreio organizado, assim como possibilitando a liberdade de decisão e a oportunidade da escolha das mulheres na sua vigilância e cuidados de saúde.

A WHO reconhece atualmente três métodos principais para o rastreio do CCU: a citologia convencional (teste de Papanicolaou), a inspeção visual com ácido acético (VIA) e o teste de deteção do DNA do HPV. Cada um destes métodos tem as suas especificidades,

vantagens e limitações. O contexto epidemiológico e os recursos de cada país determinam a escolha e a implementação de uma estratégia de rastreio adequada.

A WHO estabeleceu metas globais para a eliminação do CCU como problema de saúde pública, incluindo:

90% das meninas vacinadas contra o HPV até os 15 anos

70% das mulheres rastreadas com um teste de alta precisão aos 35 e aos 45 anos

90% das mulheres identificadas com doença cervical recebendo tratamento

Portugal implementou em 2017 um programa nacional de rastreio baseado na pesquisa de HPV, como teste de rastreio primário, para mulheres com idades dos 25 aos 60 anos.

No entanto, este programa não está totalmente implementado em todas as regiões, o que contribui para as dificuldades que o programa atravessa, com apenas 64 % das mulheres elegíveis a participarem no Rastreio em 2022, pelo que é lícito afirmar que o rastreio oportunístico (ou de conveniência) tem um papel importante na prevenção do CCU.

Os trabalhos efetuados, de que resultaram os artigos científicos publicados, expressão dos estudos levados a cabo com a experiência do programa de rastreio da ULSCB (das mulheres que foram incluídas no programa de rastreio oportunístico na Consulta de Ginecologia do Hospital da Covilhã), são a base do contributo que se pretende deixar para a melhoria da eficácia da prevenção secundária do CCU em Portugal.

Pretenderam esses estudos, apesar das suas limitações, nomeadamente a dimensão da amostra: i) avaliar o risco de HSIL em mulheres com teste de HPV-hr (não 16 e/ou 18) positivos repetidos e com citologia NILM; ii) verificar se é válido dispensar a realização da citologia nos casos de teste de HPV positivo para HPV 16 e HPV 18; iii) e desenvolver uma técnica laboratorial, que permita uma deteção adequada e pouco dispendiosa, para a determinação do genótipo do HPV em material parafinado.

Em conclusão, Portugal tem um programa de rastreio organizado baseado no teste de HPV, mas o rastreio oportunístico continua a ser uma ferramenta importante na prevenção secundária do CCU, especialmente para alcançar populações não cobertas pelo programa organizado. A integração eficaz de ambas abordagens, juntamente com a vacinação contra o HPV, afigura-se crucial para atingir as metas da OMS e reduzir significativamente a incidência e mortalidade por CCU no país.

Palavras-chave

HPV, cancro do colo do útero, rastreio, prevenção secundária

Abstract

Cancer is one of the major public health problems of this century, with significant social and economic implications. Globally, it is responsible for approximately 16.8% of all deaths and 22.8% of deaths attributed to non-communicable diseases. Cervical cancer (CC) is one of the most significant public health problems for women worldwide, being the fourth most common cancer in terms of incidence and mortality in women, with an estimated 662,301 new cases and 342,000 deaths worldwide in 2022, according to the World Health Organization (WHO) 2023. Characterized by its high incidence and mortality, particularly in low or medium Human Development Index (HDI) countries, this type of cancer is mostly associated with persistent infection by the human papillomavirus (HPV), and it is estimated that more than 90% of CC cases are attributed to high-risk genotypes of this viral agent (HPV-hr).

CC is also a significant health problem in Portugal, where in 2020, an age-standardized incidence and mortality rate of 10.7 and 3.2/100,000 women-years, respectively, were determined. According to data from The Global Cancer Observatory (IARC/WHO) 2024, the incidence and mortality rate in Portugal due to CC in 2022 was 897 and 459, respectively. CC is the 8th most common cancer among women and the 3rd most common cancer among women aged 15 to 44. The mortality rate from CC in Portugal is at very high levels of HDI.

The World Health Organization (WHO) recommends the implementation of organized, population-based screening programs as the most effective strategy for the prevention of CC. However, opportunistic screening continues to play an important role in the secondary prevention of CC in many countries, including Portugal, complementing the efforts of the screening program, overcoming difficulties in accessing organized screening, as well as enabling women's freedom of decision and the opportunity to choose their health surveillance and care.

WHO currently recognizes three main methods for CC screening: conventional cytology (Pap test), visual inspection with acetic acid (VIA) and HPV DNA detection testing. Each of these methods has its specificities, advantages and limitations, and the epidemiological context and resources of each country determine the choice and implementation of an appropriate screening strategy.

The WHO has set global goals for eliminating CC as a public health problem, including:

• 90% of girls vaccinated against HPV by age 15

• 70% of women screened with a high-precision test when they reach ages 35 and 45

• 90% of women identified with cervical disease receiving treatment

In 2017, Portugal implemented a national screening program based on the primary HPV test for women aged 25 to 60 years. However, this program is not fully implemented in all regions, which contributes to the current challenges in organized screening, resulting in only 64% of eligible women participating in screening in 2022. Thus, it is fair to say that opportunistic (or convenience) screening has an important role in preventing CC.

The work undertaken (documented in published scientific articles), is the result of the studies carried out from the ULSCB screening program (of women who were included in the opportunistic screening program at the Gynecology Consultation at Hospital da Covilhã). This work forms the basis of the contribution we intend to make towards improving the effectiveness of secondary prevention of CC in Portugal.

These studies, despite their limitations, such as the small sample size, aimed to: i) evaluate the risk of HSIL in women with repeated positive HPV-hr test (not 16 and/or 18) and with NILM cytology; ii) verify whether it is valid to waive cytology in cases of a positive HPV test for HPV 16 and HPV 18; iii) and develop a laboratory technique, which allows adequate and cheap detection, for HPV genotyping in paraffin material.

In conclusion, Portugal has an organized screening program based on HPV testing, but opportunistic screening remains an important tool in the secondary prevention of CC, especially to reach populations not covered by the organized program. Effective integration of both approaches, together with HPV vaccination, appears crucial to achieve WHO targets and significantly reduce CC incidence and mortality in the country.

Keywords

HPV, cervical cancer, screening, secondary prevention

Visão geral da tese

Esta tese está dividida em **cinco capítulos**.

Após uma breve descrição da incidência/mortalidade por cancro do colo do útero, a epidemiologia, as recomendações da OMS, a análise crítica dos principais métodos de deteção do HPV e o estado atual do rastreio do CCU em Portugal, são apresentados no **Capítulo 1**.

O **Capítulo 2** analisa os objetivos que nos propusemos estudar.

O **Capítulo 3** descreve os materiais e métodos dos estudos realizados.

O **Capítulo 4** apresenta os resultados/artigos originais publicados, emanados dos **estudos clínicos** e **laboratoriais** efetuados no âmbito do projeto desta tese.

A discussão e as perspetivas futuras são descritas no **Capítulo 5**, numa abordagem integrativa.

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Lista de Acrónimos

ACOG	American College of Obstetricians and Gynecologists
AGC	Células glandulares atípicas
APPT	Ameaça de parto pré-termo
ASC	Células pavimentosas atípicas
ASCCP	American Society for Colposcopy and Cervical Pathology
ASC-H	Atypical Squamous Cells
ASC-US	Células pavimentosas atípicas de significado indeterminado
CC	Cervical cancer
CCU	Cancro do colo do útero
CHUCB	Centro Hospitalar Universitário da Cova da Beira
CIN	Neoplasia intraepitelial do colo do útero
CIN 1	Neoplasia intraepitelial do colo do útero grau 1
CIN 2	Neoplasia intraepitelial do colo do útero grau 2
CIN 3	Neoplasia intraepitelial do colo do útero grau 3
DNA	Ácido desoxirribonucleico
DS	Dupla coloração imunocitoquímica dos biomarcadores p16/Ki67
FCS	Faculdade de Ciências da Saúde
FDA	Food and drug administration
HC2	Captura híbrida 2
HDI	Human Development Index
HIV	Vírus da imunodeficiência adquirida
HSIL	High-grade intraepithelial lesion
HPV	Papilomavírus humano
HPV-hr	Papilomavírus humano de alto risco
IARC	International Agency for Research on Cancer
IDH	Índice de desenvolvimento humano
LAST	Lower anogenital squamous terminology project
LBC	Citologia em meio líquido
LSIL	Low-grade intraepithelial lesion
NILM	Negative for intraepithelial lesion or malignancy
OMS	Organização Mundial da Saúde
PCR	Polymerase chain reaction
PNV	Plano Nacional de Vacinação
RAA	Região Autónoma dos Açores
RAM	Região Autónoma da Madeira
RCCU	Rastreio do cancro do colo do útero
RNA	Ácido ribonucleico
RNAm	Ácido ribonucleico mensageiro
SPSS	Pacote Estatístico para as Ciências Sociais
ULSCB	Unidade Local de Saúde da Cova da Beira
USPSTF	United States Preventive Services Task Force
VIA	Inspeção visual com ácido acético
VPN	Valor preditivo negativo
VPP	Valor preditivo positivo
WHO	World Health Organization

A investigação realizada para esta tese, levou à publicação dos seguintes artigos em revistas internacionais indexadas, com revisão por pares:

Caeiro V, Nunes S, Esteves B, Moutinho-Fonseca J (2021) Repeated Positive Cervical HPV Testing and Absent or Minor Cytology Abnormality at Pap Smear. What is the Next Step? Asian Pac J Cancer Prev. 2021 Jun 1;22(6):1907-1912. doi: 10.31557/APJCP.2021.22.6.1907. PMID: 34181350; PMCID: PMC8418856.

Caeiro V, Esteves B, Fonseca-Moutinho J (2023) HPV testing for cervical cancer screening: Should reflex cytology be performed after a positive test for HPV 16 and 18? Cancer Treat Res Commun. 2023; 36:100729. doi: 10.1016/j.ctarc.2023.100729. Epub 2023 Jun 14. PMID: 37352587.

Micaela Almeida*, **Vitor Caeiro***, Diana Costa, Lara Silva, Cíntia Sousa, Paula Pestana, Sofia Campelos, João Vale, Ana Cristina Ramalhinho, José Fonseca-Moutinho and Luiza Breitenfeld (2024) High-Risk HPV Detection in Paraffin-Embedded Tissue from Cervical Lesions. *Pharmaceuticals* 2024, *17*(9), 1201; doi: 10.3390/ph17091201

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Publicações científicas a que deu colaboração:

Anexo 4 (submetido para publicação): Rita Sousa, MD, José Fonseca Moutinho, MD, PhD; **Vitor Caeiro**, MD; Mónica Barros, MD; Helena Nascimento, MD; Sofia Pereira, MD MSc; Madalena Ponte, MD; Teresa Rebelo, MD MSc; Isabel Saavedra, MD MSc; Helena Solheiro, MD; Fábio Gomes, MD MSc; Fernanda Loureiro, MD MSc; Ana Rita Goes, MD, PhD; Patrícia Soares (2025) Patterns in Invasive Cervical Cancer Incidence: Exploring Factors Contributing to Higher Rates

Publicações em atas de Congressos Nacionais:

Poster: "A persistência de teste de HPV positivo, com alterações citológicas minor. Qual a conduta seguinte?", em 197ª Reunião da SPG- Ginecologia Oncológica – Coimbra 14 e 15/01/2022

Poster: "Rastreio do cancro do colo do útero por teste de HPV. Haverá vantagem em fazer citologia reflexa nos casos de HPV 16 e 18 positivos?", em XV – Congresso Português de Ginecologia – Estoril, 2,3 e 4 de junho/2022

A investigação realizada para esta tese, conduziu à seguinte conferência:

"HPV e o Futuro" – Covilhã, 24/03/2019 na FCS da UBI

Capítulo 1 – Introdução

Parte do conteúdo deste capítulo foi publicado originalmente em:

HPV testing for cervical cancer screening: Should reflex cytology be performed after a positive test for HPV 16 and 18?

Vitor Caeiro a, *, Bruno Esteves b, José Fonseca-Moutinho a

a *Health Sciences Research Centre (CICS), Faculty of Health Sciences, University of Beira Interior (UBI), Covilhã, Portugal*

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E-mail address: vcaeiro@fcsaude.ubi.pt (V. Caeiro).

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Cancer Treatment and Research Communications 36 (2023)

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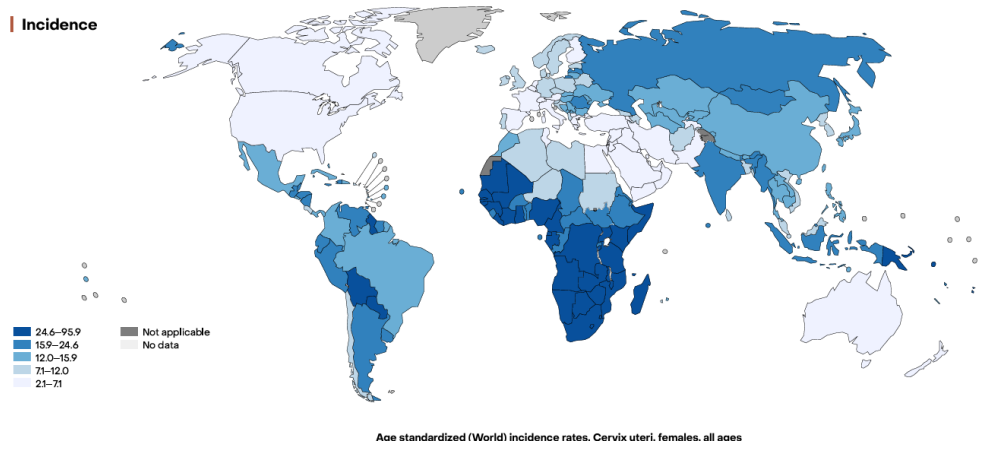
1.1 - Epidemiologia do cancro do colo do útero

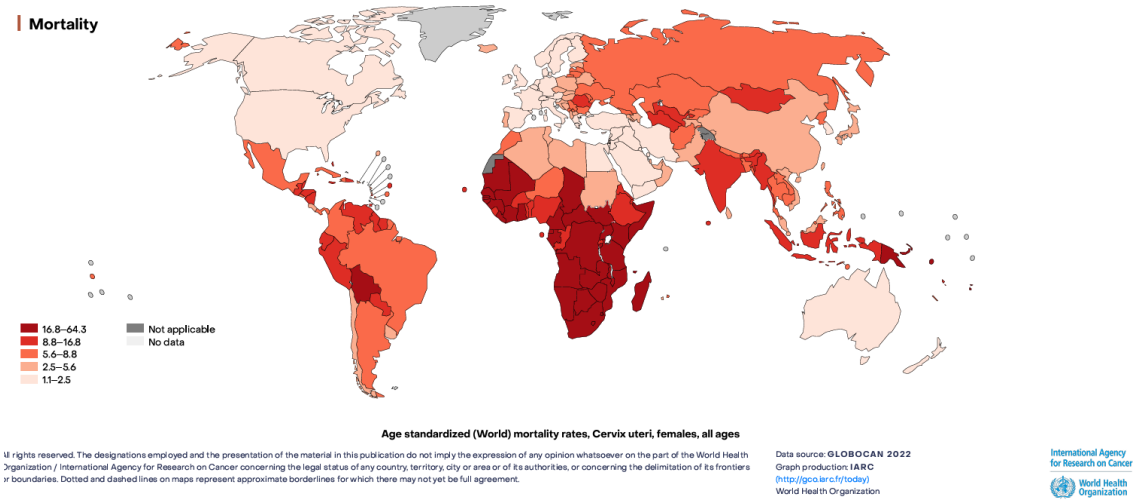
O cancro do colo do útero (CCU) é um dos problemas de saúde pública mais significativos para a população feminina em todo o mundo [1].

Caracterizado pela sua elevada incidência (Figura 1) e mortalidade (Figura 2), particularmente em países de Índice de Desenvolvimento Humano (IDH) baixo ou médio, este tipo de cancro está maioritariamente associado à infeção persistente pelo vírus do papiloma humano (HPV), e estima-se que mais de 90% dos casos de CCU sejam atribuídos a este agente viral [2][3].

O IDH é um índice composto por três dimensões básicas do desenvolvimento humano: uma vida longa e saudável (com base na esperança de vida à nascença), educação (com base na média e anos esperados de escolaridade) e um padrão de vida digno (com base no rendimento nacional bruto per capita). Os níveis de desenvolvimento dos países podem ser considerados de acordo com quatro níveis de IDH: IDH baixo, médio, alto e muito alto [2].



Figure/Figura 1 - Taxa padronizada por idade (mundial) por 100.000, incidência, em 2022 (cancro do colo do útero). Fonte: GLOBOCAN 2022.

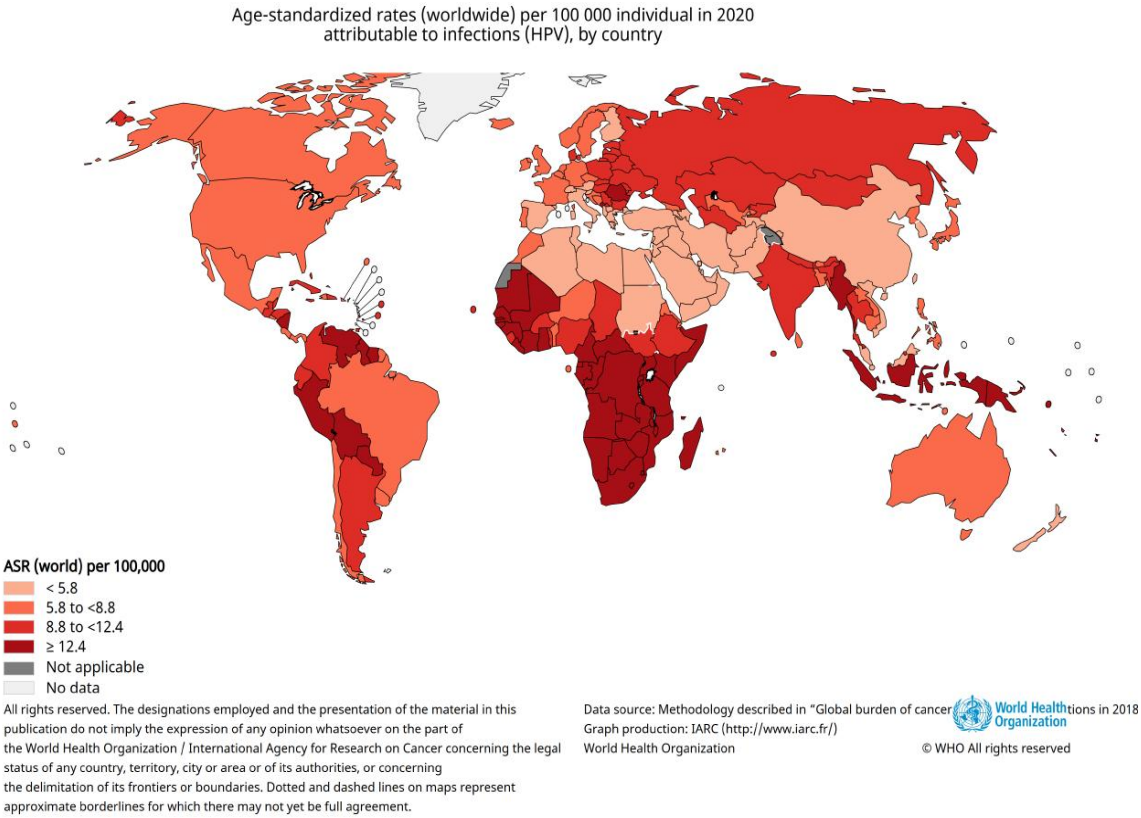


Figure/Figura 2 - Taxa padronizada por idade (mundial) por 100.000, mortalidade, em 2022 (cancro do colo do útero). Fonte: GLOBOCAN 2022.

Globalmente, o CCU é o quarto tipo de cancro mais comum nas mulheres,[4] o que realça a importância de estratégias de rastreio eficazes e amplamente implementadas para detetar lesões pré-cancerosas e reduzir a progressão para cancro invasivo[5].

Rótulo	Código do câncer	Código do país (ISO/ONU)	Código alfa-3	Sexo	ASR (Mundo)	Taxa bruta	Risco cumulativo	Total
África	23	903	N / D	0	26.4	17.9	-	125 699
América Latina e Caribe	23	904	N / D	0	15.1	18.7	-	63 171
América do Norte	23	905	N / D	0	6.4	8.3	-	15 654
Europa	23	908	N / D	0	10.6	15.1	-	58 219
Oceânia	23	909	N / D	0	9.6	11.3	-	2 476
Ásia	23	935	N / D	0	13.9	17,5	-	397 082

Table/Tabela 1 - Taxas de incidência padronizadas por idade (mundial), colo do útero, mulheres, todas as idades. Fonte: GLOBOCAN 2022.

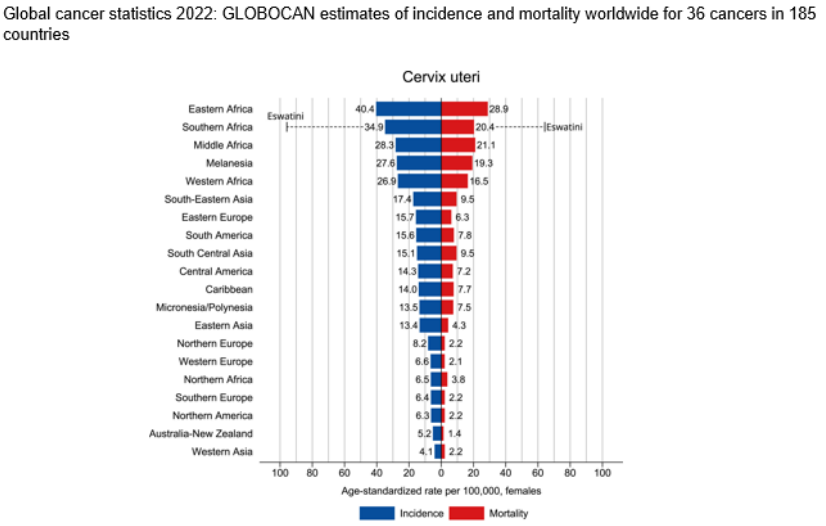


Figure/Figura 3 - Taxas padronizadas por idade (em todo o mundo) por 100.000 indivíduos em 2020, atribuíveis a infeções (HPV), por país. Fonte: GLOBOCAN 2022.

O CCU é uma das principais causas de mortalidade entre as mulheres, sobretudo em países sem programas de rastreio generalizados [5]. Em 2022, cerca de 662 301 mulheres foram diagnosticadas com CCU em todo o mundo e cerca de 342 000 mulheres morreram devido à doença [4].

A alta taxa de mortalidade do CCU a nível mundial poderia ser reduzida através de uma abordagem abrangente que inclua prevenção, diagnóstico precoce, programas eficazes de rastreio e tratamento [6].

Aproximadamente 90% das mortes por CCU ocorreram em países de IDH baixo e médio, com uma taxa de mortalidade 18 vezes superior à dos países de IDH alto e muito alto, destacando a disparidade no acesso aos cuidados de saúde e nas estratégias de prevenção [7].



Figure/Figura 4 - Taxas de incidência e mortalidade específicas da região padronizadas por idade para cancro do colo em 2022. Fonte: GLOBOCAN 2022.

Rótulo	Código do câncer	Código do país (ISO/ONU)	Código alfa-3	Sexo	ASR (Mundo)	Taxa bruta	Risco cumulativo	Total
África	23	903	N / D	0	17.6	11,5	-	80 614
América Latina e Caribe	23	904	N / D	0	7.7	9.9	-	33 514
América do Norte	23	905	N / D	0	2.2	3.6	-	6 692
Europa	23	908	N / D	0	3.9	7.0	-	26 950
Oceânia	23	909	N / D	0	4.5	6.0	-	1 309
Ásia	23	935	N / D	0	6.7	8.8	-	199 795

Table/Tabela 2 - Taxas de mortalidade padronizadas por idade (mundial), colo do útero, mulheres, todas as idades. Fonte: GLOBOCAN 2022

O CCU é um problema de saúde significativo também em Portugal, onde em 2020, foi determinada uma incidência e mortalidade padronizada para a idade de 10,7 e 3,2/100 000 mulheres ano, respetivamente [8][10].

De acordo com os dados do The Global Cancer Observatory (IARC/OMS) 2024, a Incidência e Mortalidade em Portugal por CCU em 2022, foi de 897 e 459, respetivamente, tendo sido nesse ano, o 8.º cancro mais frequente entre as mulheres e o 3.º cancro mais frequente entre as mulheres entre os 15 e os 44 anos [9]. Estes números correspondem a uma taxa de incidência de 6,8 por 100.000 mulheres e a uma taxa de mortalidade de 2,9 por 100.000 mulheres. O CCU representou 1,5% de todos os novos casos de cancro e 1,3% de todas as mortes por cancro no país nesse ano [9].

Segundo a OMS, a infeção pelo vírus do papiloma humano (HPV) é a infeção viral do aparelho reprodutor mais frequente [11]. A maioria dos indivíduos sexualmente ativos será infetada num dado momento da sua vida (podendo ocorrer infeções repetidas), mais frequentemente pouco tempo após o início da atividade sexual [11][12].

Há uma relação bem estabelecida entre a infeção por HPV e a etiopatogenia do CCU, podendo ser considerado que esta é condição necessária, mas não suficiente, para o desenvolvimento de CCU [13]. Virtualmente, todos os CCU têm a sua origem numa infeção persistente causada por estirpes oncogénicas (denominadas de alto-risco) de vírus do papiloma humano (HPV-hr), mas apenas uma pequena proporção dessas infeções persiste, evoluindo para uma lesão pré-cancerígena, sendo que esta persistência depende não só das diferenças genéticas existentes entre os vários genótipos de HPV, como também de outros fatores, nomeadamente a idade precoce de início da atividade sexual (coitarca), múltiplos parceiros sexuais ou parceiro com múltiplos parceiros sexuais (promiscuidade sexual), a não utilização de preservativo, a multiparidade, o tabagismo, a contraceção hormonal prolongada, o compromisso da imunidade, outras infeções sexualmente transmissíveis, estados de carência nutricional e fatores genéticos [11]. Para além disso, a maioria das infeções regride espontaneamente alguns meses após a sua aquisição, ou seja, nem todas as infeções por HPV irão culminar em CCU [11][12].

Por este motivo, o rastreio baseia-se principalmente na deteção de HPV ou de lesões secundárias à infeção. É necessário, no entanto, ter em conta que o intervalo de tempo entre a infeção por HPV e a evolução para cancro invasivo é, habitualmente, longo (entre 15 a 20 anos em mulheres imunocompetentes e entre 5 a 10 anos em mulheres com diminuição da imunidade) [11].

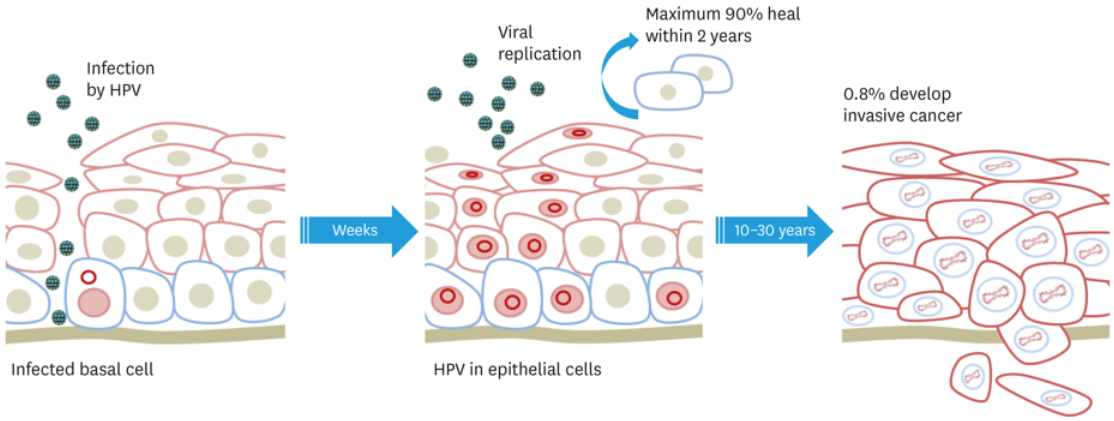
São conhecidos mais de 200 tipos de HPV, classificados em 5 diferentes géneros, alfa, beta, gama, mu e nu [14][15]. Os tipos envolvidos na carcinogénese cervical estão incluídos no grupo alfa [14].

Os HPV pertencentes a este género podem ser divididos em dois grupos: os de baixo risco e os de alto risco [12][15]. Os de baixo risco estão associados principalmente a condilomas acuminados anogenitais, nomeadamente os genótipos 6 e 11, responsáveis por cerca de 90% destes casos [15]. Os HPV de alto risco (HPV-hr) incluem os classificados pela IARC como carcinogénicos do grupo 1 (“carcinogénicos para humanos”), nomeadamente os genótipos 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 e 59, [12][15][16], que podem estar associados a neoplasias malignas do colo do útero, da

vagina, da vulva, do pénis, do ânus e da cabeça e pescoço [12][15]. Os genótipos 16 e 18 são os mais frequentemente responsáveis por CCU, sendo detetados em cerca de 70% dos casos [12][14][15].

A evolução de uma lesão pré-cancerígena para uma neoplasia invasiva do epitélio do colo do útero é um processo que pode levar vários anos ou décadas, o que permite a utilização de métodos de prevenção para a deteção de lesões pré-invasivas [8][10].

Globalmente, o CCU é o quarto tipo de cancro mais comum nas mulheres,[4] o que realça a importância de estratégias de rastreio eficazes e amplamente implementadas para detetar lesões pré-cancerosas e reduzir a progressão para cancro invasivo[5].



Figure/Figura 5 - Infeção e carcinogénese pelo vírus do papiloma humano de alto risco (HPV-Hr). Fonte: [151]

Estão disponíveis dois tipos de estratégias de prevenção e controlo do CCU, ambas consideradas custo-efetivas na redução da carga de doença da população (i.e., *best buy* pela OMS) [11][12]: 1) Prevenção primária através da Vacinação contra o HPV; 2) Prevenção secundária, preferencialmente através de programa organizado de rastreio do CCU.

Deve ser feita prevenção primária da infeção por HPV e dos cofatores que aumentam o risco de CCU, através da implementação de estratégias para influenciar alterações comportamentais tendo em conta os fatores de risco (uso de preservativos, educação sexual) e da vacinação, antes do início da vida sexual, contra a infeção pelo HPV [5].

A vacinação contra a infeção por HPV, recomendada pela OMS [19], está contemplada em Portugal no Plano Nacional de Vacinação (PNV) para o sexo feminino desde 2008, ano em que foi introduzida a vacina tetravalente, concebida para os genótipos 6, 11, 16, 18, posteriormente substituída pela vacina nonavalente, com proteção mais alargada, para os genótipos 6, 11, 16, 18, 31, 33, 45, 52, 58. Posteriormente, em 27/09/2020, a DGS emitiu a Norma nº 018/2020, de acordo com o Despacho nº 12434/2019, publicado no D. R., 2ª série – nº 250 de 30 de dezembro de 2019, que atualiza o PNV, o qual passa a incluir no esquema vacinal recomendado, o alargamento ao sexo masculino, aos 10 anos de idade, da vacinação contra infeções por HPV, incluindo os genótipos causadores de condilomas anogenitais [19].

Efetuamos prevenção secundária com deteção precoce de lesões pré-malignas, através da implementação de programas de rastreio, preferencialmente organizados e de base populacional, que preveem a convocação das mulheres dos grupos-alvos identificados, de acordo com uma calendarização definida (sendo a cobertura populacional e a frequência dos exames cruciais para maximizar os benefícios preventivos) [5][11].

Em Portugal, em 2017 foi implementado um programa de rastreio organizado do CCU, de âmbito nacional, [28] que foi aperfeiçoado em 2024 pela **Norma Número: 09/2024** de **17/10/2024.**

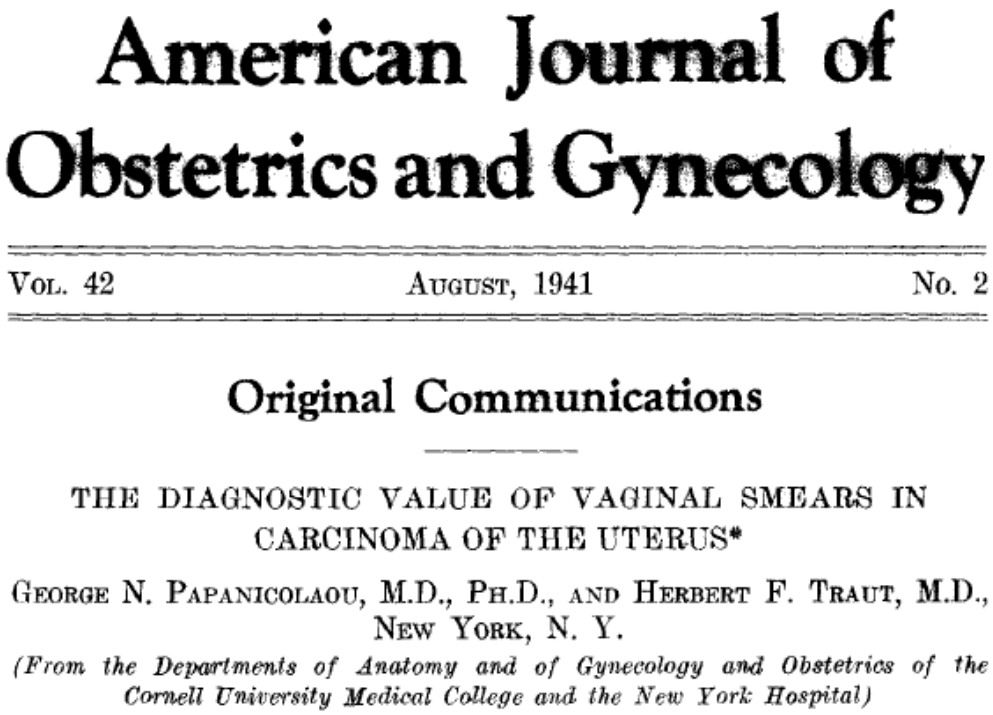
A OMS e a Comissão Europeia recomendam a realização de rastreio às mulheres com 30 ou mais anos de idade [22]; a Comissão Europeia recomenda que o RCCU inclua mulheres até aos 65 anos [23]. No entanto, por consenso do Painel de Peritos da presente Norma, recomenda-se manter o Programa de rastreio até aos 69 anos, justificado pelo facto de a vacinação contra o HPV não incluir a população com idade superior a 50 anos, a existência de cobertura populacional do Programa de rastreio de CCU inferior a 50% até 2021 e por, em 2018, 19% dos casos de CCU terem sido identificados em mulheres com idades compreendidas entre os 60-70 anos [24]. Não existe ainda evidência sólida sobre o custo-efetividade do alargamento do Programa de rastreio do CCU para a faixa etária dos 65 aos 70 anos, nos países da União Europeia. No entanto, existe evidência de base epidemiológica, em Portugal, que sustenta este alargamento etário [24].

Reconhecendo que esta doença é amplamente prevenível, a Organização Mundial de Saúde (OMS) lançou, em 2020, uma "Estratégia Global para a Eliminação do Cancro do Colo do Útero como Problema de Saúde Pública"[7]. Esta iniciativa estabelece metas ambiciosas a atingir até 2030, com base na abordagem 90-70-90: vacinar 90% das

raparigas contra HPV antes dos 15 anos, garantir que 70% das mulheres são rastreadas com métodos eficazes aos 35 e aos 45 anos, e garantir que 90% das mulheres com doenças cervicais recebem tratamento adequado. O objetivo final da estratégia é reduzir a incidência do CCU para menos de 4 casos por 100.000 mulheres por ano, eliminando-o como um problema de saúde pública. Para alcançar esta meta, é essencial implementar medidas integradas, que incluem a educação comunitária, o reforço dos sistemas de saúde, o aumento do acesso a serviços de prevenção e tratamento, e a monitorização rigorosa dos progressos [7]. Esta apresentação irá explorar os fundamentos, as metas e as ações propostas pela OMS, destacando a sua relevância na transformação da saúde global até 2030 [26].

A redução da incidência e da mortalidade por CCU só será possível através da adoção de medidas de prevenção primária e secundária [27]. A prevenção primária da infeção por HPV e dos cofatores que aumentam o risco de CCU, deve ser assegurada através de estratégias para influenciar alterações comportamentais, tendo em conta os fatores de risco (uso de preservativos, educação sexual) e da vacinação, especialmente antes do início da vida sexual, contra a infeção pelo HPV [27]. A prevenção secundária comporta a deteção precoce de lesões pré-malignas, através da implementação de programas de rastreio organizados que implicam a convocação das mulheres dos grupos-alvos identificados, de acordo com uma calendarização, com controlo de qualidade, sendo a cobertura populacional e a frequência dos exames cruciais para maximizar os benefícios preventivos [27].

Na década de 1940, o CCU era uma das principais causas de morte das mulheres em idade fértil nos Estados Unidos. Geórgios Papanikoláou, um imigrante médico, começou a sua carreira académica estudando os ciclos reprodutivos das cobaias (porquinhos da Índia). Depois de se mudar para os Estados Unidos, ocupou um cargo no Departamento de Anatomia da Universidade de Cornell. Mudou o seu foco de estudo para a fisiologia humana e começou a colaborar com o patologista ginecológico Herbert Traut. Enquanto trabalhavam juntos na Universidade de Cornell, publicaram no American Journal of Obstetrics and Gynecology, no seu Vol. 42 no Nº2, em agosto de 1941 o artigo "The diagnostic value of vaginal smears in carcinoma of the uterus" e mais tarde no, Yale J Biol Med .1943 Jul;15(6):924, um artigo sobre "*Diagnosis of Uterine Cancer by the Vaginal Pap Smear*". Este importante trabalho descrevia em pormenor a forma como as células vaginais e cervicais normais e anormais podiam ser observadas ao microscópio e como deviam ser classificadas. Pouco tempo depois, o exame de Papanicolaou tornou-se a norma de ouro no rastreio do CCU [29].



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THE DIAGNOSTIC VALUE OF VAGINAL SMEARS IN CARCINOMA OF THE UTERUS*

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Figure/Figura 6 - "The diagnostic value of vaginal smears in carcinoma of the uterus"

A implementação de programas de rastreio de base populacional para o CCU tem tido um impacto significativo na redução das taxas de incidência e mortalidade em várias regiões do mundo [30]. Nos países com IDH muito alto e alto, onde foram estabelecidos programas de rastreio organizados desde meados do século XX, observou-se uma redução substancial da mortalidade [31]. Contrariamente, em regiões com recursos limitados (com IDH medio e baixo) e sem programas de rastreio estruturados, o CCU continua a ser uma das principais causas de morte entre as mulheres, como acontece em áreas da África Subsariana, do Sudeste Asiático e da América Latina [32].

Nos Estados Unidos, onde não existe um programa nacional de rastreio organizado do CCU, a prática regular, oportunista, do rastreio citológico, permitiu que a taxa de mortalidade por CCU diminuísse mais de 60% desde a década de 1950. Atualmente, é o 14.º cancro mais comum entre estas mulheres, o que indica uma redução acentuada tanto da incidência como da mortalidade [33].

Em 2010, nos Estados Unidos, cerca de 12.000 mulheres foram diagnosticadas com CCU, resultando em 4.000 mortes, aproximadamente. Entre 2002 e 2012, a taxa de

incidência de CCU diminuiu 1,3% e as mortes por CCU diminuíram 0,9%. Em 2014, as mulheres hispânicas registaram a taxa mais elevada de diagnósticos de CCU, ao passo que as mulheres afro-americanas registaram a taxa mais elevada de mortalidade por esta doença [27], o que sugere desigualdade social nos cuidados de saúde, aspeto que favorece a implementação de um programa nacional de rastreio organizado do CCU, visto que, ao ser gratuito, se torna mais acessível a grupos populacionais que, atualmente, não têm possibilidade social ou financeira para participar [7].

Desde a introdução dos primeiros métodos de rastreio na década de 1940, a história do rastreio do CCU tem sido marcada por avanços científicos e tecnológicos que permitiram a deteção precoce e o tratamento de lesões pré-cancerosas, reduzindo assim a sua incidência e a mortalidade [35]. O teste de Papanicolaou (ou citologia cervical), foi o primeiro método de rastreio amplamente utilizado e demonstrou uma eficácia significativa na redução da mortalidade nos países onde foi sistematicamente implementado. Posteriormente, a verificação que a infeção pelo HPV de alto risco era condição necessária na carcinogénese do colo do útero, conduziu ao desenvolvimento de técnicas baseadas na deteção do DNA do HPV, o que trouxe novos horizontes ao rastreio, permitindo uma maior sensibilidade na identificação de infeções de alto risco e na estratificação do risco [36].

O rastreio do CCU é uma medida de prevenção secundária, cuja finalidade é identificar as mulheres assintomáticas com lesões pré-cancerosas, para permitir o diagnóstico atempado e o tratamento mais adequado antes que o cancro se desenvolva. Os testes utilizados no rastreio devem ser sensíveis, reprodutíveis, facilmente realizados e geridos pelos cuidados primários de saúde [38]. A citologia cervical (teste de Papanicolaou) foi o esteio do rastreio, durante décadas, mas o teste de HPV assumiu um papel cada vez mais importante à medida que se aprofundou o papel da infeção por HPV no desenvolvimento do CCU [36]. O objetivo de um teste de rastreio é separar com precisão, os indivíduos com risco significativo de doença, daqueles com baixo risco de doença e minimizar os resultados falso-negativos [37]. Com base nesses parâmetros, o teste de HPV é claramente superior ao teste de Papanicolaou, como teste de rastreio para o CCU, porque deteta muito mais lesões pré-cancerosas por cada triagem. Adicionar a citologia ao teste de HPV (co-teste) aumenta ligeiramente o número de casos detetados, mas à custa de mais resultados falso-positivos e procedimentos mais invasivos (colposcopias e biópsias) [38].

1.2 - As Recomendações da Organização Mundial de Saúde para o Rastreio do Cancro do Colo do Útero

A OMS tem incentivado a utilização de uma abordagem integrada de rastreio, adaptada às necessidades e recursos de cada região, promovendo a combinação de métodos, como a utilização de testes de DNA do HPV seguidos de citologia em populações de alto risco. Para além disso, recomenda a implementação de políticas de saúde pública que incluam campanhas de sensibilização, educação e formação dos profissionais de saúde, bem como o acesso facilitado à vacinação contra o HPV, que se tem mostrado eficaz na redução da prevalência da infeção e, consequentemente, das lesões pré-cancerosas [39].

A evolução dos métodos de rastreio está agora refletida nas recomendações da OMS, que incluem não só a citologia, mas também testes moleculares para a deteção do HPV. Estes métodos são reconhecidos pela OMS pela sua elevada sensibilidade, o que contribui para a deteção precoce e eficaz de lesões pré-cancerosas. No entanto, cada método tem vantagens e limitações que devem ser consideradas no contexto das necessidades e dos recursos de cada país. Para uma compreensão aprofundada, é essencial examinar a **evolução histórica destes métodos de rastreio,** desde os seus primórdios até aos avanços recentes e aos desafios que subsistem na sua implementação [40].

Durante várias décadas, o teste de Papanicolaou foi o método mais utilizado e reconhecido para o rastreio do CCU. A sua simplicidade e custo relativamente baixo, juntamente com a sua alta capacidade de detetar alterações celulares pré-cancerosas (alta especificidade), tornaram-no um instrumento essencial em saúde pública [41]. No entanto, o método tem limitações, incluindo uma sensibilidade moderada e a necessidade de infraestruturas laboratoriais e de pessoal especializado para analisar as amostras [38]. Atendendo que a infeção pelo HPV é condição necessária para o desenvolvimento das lesões, a deteção do DNA do HPV surgiu como um método complementar e, em alguns casos, como uma alternativa mais sensível e adequada, especialmente em contextos em que a prevalência da infeção pelo HPV é elevada, na medida em que pode ser automatizado e tem uma sensibilidade significativamente superior à citologia [41][42].

A Organização Mundial de Saúde (OMS) reconhece atualmente três métodos principais para o rastreio do CCU: a citologia convencional (teste de Papanicolaou), a inspeção visual com ácido acético (VIA) e o teste de deteção do DNA do HPV. Cada um destes métodos tem as suas especificidades, vantagens e limitações. O contexto epidemiológico e os recursos de cada país determinam a escolha e a implementação de uma estratégia

de rastreio adequada. Além disso, a OMS propõe estratégias combinadas e recomenda a vacinação contra o HPV como medida preventiva complementar ao rastreio, numa tentativa de alcançar a eliminação do CCU como um problema de saúde pública [38].

A inspeção visual com ácido acético (VIA) representa uma alternativa simples e de baixo custo, particularmente adequada para regiões com infraestruturas limitadas, onde o acesso a técnicas laboratoriais avançadas é restrito. A VIA permite o rastreio em tempo real e uma resposta rápida, o que a torna uma opção viável para programas de rastreio baseados em unidades móveis e de proximidade. No entanto, a VIA tem uma especificidade mais baixa em comparação com outros métodos e depende da experiência do profissional de saúde, o que pode comprometer a exatidão do diagnóstico e o sobre tratamento [43][44].

Com o avanço dos conhecimentos sobre a relação entre o HPV e o CCU, surgiram métodos de rastreio baseados na deteção do DNA e do RNA do HPV. Estes testes moleculares, como o Hybrid Capture 2 e os testes de PCR em tempo real (como o Cobas HPV e o Aptima HPV), são atualmente recomendados pela OMS como métodos de rastreio primário para mulheres a partir dos 30 anos. A principal vantagem dos testes moleculares é a sua maior sensibilidade (Table/Tabela 3) em comparação com a citologia, permitindo identificar precocemente as infeções por HPV de alto risco, antes do desenvolvimento das lesões [41][42][39].

A aplicação dos testes de pesquisa do DNA e RNA do HPV de alto risco foi um avanço considerável atendendo à sua elevada sensibilidade, mas a prevalência da infeção é significativamente superior à das lesões de alto grau, o que implica que o seu uso como teste isolado, leve a que muitas mulheres sem doença significativa sofram do processo de diagnóstico sem necessidade, com todas as consequências que daí advém.

Table/Tabela 3 - Precisão dos testes de rastreio do CCU (deteção de lesões de HSIL)

Teste	Sensibilidade (%)	Especificidade (%)
Citologia	62,5-72,9	90,3-96,6
Inspeção visual com ácido acético	74,2-79,4	85,2-85,8
Inspeção visual com iodo de Lugol	89,7-93,4	85,4
Teste de DNA do HPV	94	88

(retirado de: Rajaram S, Gupta B. Screening for cervical cancer: Choices & dilemmas. Indian J Med Res. 2021 Aug;154(2):210-220. doi: 10.4103/ijmr.IJMR_857_20. PMID: 34854432; PMCID: PMC9131755.)

Na tentativa de melhorar a efetividade do rastreio, foi necessário desenvolver novos testes de triagem que permitem estratificar o risco e indicar as mulheres que verdadeiramente necessitam da avaliação em colposcopia, nomeadamente a dupla coloração imunocitoquímica dos biomarcadores p16/Ki67 (DS) [43][44].

A OMS reconhece também a utilidade do teste da dupla marcação imunocitoquímica, que deteta a expressão das proteínas p16 e Ki-67 nas células cervicais infetadas com HPV de alto risco, indicando atividade oncogénica. Este tipo de teste é utilizado em situações em que é necessário confirmar se a infeção por HPV está associada a um risco elevado de progressão [45][46].

A DS apresenta sensibilidade e especificidade superiores ao co-teste, para a deteção de lesões precursoras, e o valor preditivo positivo (VPP) e valor preditivo negativo (VPN) são igualmente elevados. A DS constitui um ótimo auxílio para a estratificação de risco nas mulheres com positividade para o HPV e estima-se que reduz para metade o número de colposcopias necessárias [43][44].

A combinação do teste HPV-hr com a citologia (co-teste), decide quais as mulheres que devem ser referenciadas à colposcopia [47]. No entanto, muitas das colposcopias são dispensáveis, não sendo viável nem eficiente orientar todas as mulheres HPV positivas, porque a maioria não apresenta lesões precursoras para o CCU e, evita-se um número considerável de biópsias e tratamentos injustificados. [47][48].

De facto, a DS tem a capacidade de reduzir as referenciações desnecessárias à colposcopia [44] pela elevada sensibilidade e especificidade para a deteção de lesões precursoras, revelando-se os respetivos valores preditivo negativo e preditivo positivo superiores [45].

Por conseguinte, contribuiu para uma mudança de paradigma, tornando o rastreio do CCU mais eficiente, com redução da morbilidade induzida pela realização de intervenções, possibilitando uma diminuição do custo global no seguimento das mulheres e minimizando a ansiedade e iatrogenia associadas [46].

Outro teste consiste na deteção do mRNA das oncoproteínas E6 e E7 dos HPV de alto risco, essenciais para a transformação maligna das células cervicais, traduzindo o aumento da sua expressão, lesões mais graves ou infeções persistentes por HPV, com maior risco de progressão para lesões de alto grau e carcinoma, pelo que a sua deteção poderá ser utilizada como marcador no rastreio do CCU [52][53][54].

A correlação entre a deteção do mRNA E6/E7 e a gravidade das lesões já foi demonstrada em alguns estudos [55][56][57].

A avaliação da metilação do DNA, tanto do genoma do HPV como do hospedeiro, tem sido proposta como uma outra forma de estratificar o risco de progressão das lesões por HPV [56]. A alteração da metilação do DNA, com remoção ou adição de grupos metil à citosina dos dinucleótidos CpG, regulando a expressão genética, [58] parece contribuir para a progressão das lesões intraepiteliais cervicais, provocando instabilidade genómica, inativação de genes supressores tumorais e ativação de oncogenes [59].

Outros estudos, avaliaram a alteração da expressão de microRNAs na evolução do CCU, relativamente ao tecido cervical normal, mostrando diferenças significativas numa grande variedade de microRNAs, razão pela qual se começou a investigar a sua possível utilidade no rastreio do CCU [61][62][64]. Os microRNAs são pequenas moléculas de RNA, não codificantes, cuja função é regular a expressão de genes através da ligação à região 3'UTR de mRNAs alvo, levando à sua destruição ou à inibição da tradução [51][63][65].

Estudos realizados através de imunocitoquímica e imunohistoquímica demonstraram que a expressão da proteína BIRC5, uma proteína inibidora da apoptose, cuja presença em células superficiais do colo do útero traduz uma alteração do ciclo celular, [66][67] está significativamente relacionada com a gravidade das lesões do colo do útero, sendo

tanto maior quanto mais grave a lesão [68][69]. No entanto, a sua sensibilidade e especificidade como método de rastreio do CCU, são relativamente baixas [70].

Existem ainda estudos que avaliaram a expressão de duas proteínas nucleares, nomeadamente os genes TOP2A e MCM2, de expressão prolongada na fase S do ciclo celular, [71][73] que está aumentada em vários carcinomas em humanos, nomeadamente no carcinoma cervical, pelo que foram propostas como marcadores moleculares no CCU, trazendo potenciais vantagens ao rastreio [71][72][74].

Metas propostas pela OMS para a eliminação do CCU

A 17 de novembro de 2020, a Organização Mundial de Saúde lançou uma estratégia global para a eliminação do CCU assente em três pilares (vacinação, rastreio e tratamento) com o objetivo de assegurar a vacinação completa de 90% das raparigas até aos 15 anos, o rastreio de 70% das mulheres entre os 35 e os 45 anos de idade e o tratamento de 90% de mulheres diagnosticadas com lesões pré-invasivas ou cancro invasivo. O alcance destas metas resultaria numa redução na incidência de CCU de 42% em 2045 e 97% em 2120 [7].

A estratégia global para eliminar o CCU propõe:

- uma visão de um mundo onde o CCU é eliminado como um problema de saúde pública;

- um limiar de 4 por 100 000 mulheres/ano para eliminação como problema de saúde pública;

- as seguintes metas 90–70–90 que devem ser cumpridas até 2030 para que os países estejam no caminho da eliminação do CCU:

90% das raparigas totalmente vacinadas com a vacina contra o HPV até aos 15 anos

70% das mulheres são rastreadas com um teste de alto desempenho até aos 35 anos de idade e novamente aos 45 anos de idade

90% das mulheres identificadas com doença cervical recebem tratamento (90% das mulheres com lesões pré-cancerosas tratadas e 90% das mulheres com CCU invasivo controlado).

- Foi criado um modelo matemático que ilustra os benefícios provisórios de atingir as metas 90–70–90 até 2030 em países de IDH baixo e médio:

a taxa média de incidência de CCU cairá 42% até 2045 e 97% até 2120, evitando mais de 74 milhões de novos casos de CCU;

o número cumulativo médio de mortes por CCU evitadas será de 300.000 até 2030, mais de 14 milhões até 2070 e mais de 62 milhões até 2120.

A estratégia global da Iniciativa da OMS para a Eliminação do CCU consiste em reduzir as taxas de incidência de CCU para menos de 4 por 100.000 mulheres/ano neste século, eliminando assim a doença como problema de saúde pública [49].

De acordo com as estimativas do estudo levado a cabo por Brisson M, Kim JJ, Canfell K, et al., [79] a maioria dos países (exceto 10, todos no Mediterrâneo Oriental) tinham em 2022 taxas de incidência de CCU relativamente elevadas, pelo que os estudos de modelização realizados indicam que o objetivo de eliminação do CCU como problema de saúde pública (<4 por 100.000 mulheres/ano) poderá não ser alcançado antes do final do século nestes países com IDH baixo e médio, sem aumentar significativamente as intervenções preventivas e curativas, incluindo o rastreio e a vacinação contra o HPV.

É encorajador que existam evidências promissoras que apoiam o potencial de uma **abordagem de auto-colheita** para aumentar a participação no rastreio de mulheres pouco ou nunca rastreadas e a eficácia de uma vacina de dose única até aos 14 anos para facilitar a adesão aos programas de vacinação [75] [76] [77][123].

Após o lançamento pela OMS da Estratégia Global, um painel de especialistas reuniu-se para definir as principais áreas de enfoque para aumentar o acesso ao rastreio e ao tratamento, a fim de se alcançarem as metas definidas para 2030. Uma das áreas de enfoque acordadas foi a atualização das atuais diretrizes da OMS de 2013 para a prevenção, o rastreio e tratamento de lesões pré-cancerosas do CCU e a simplificação dos algoritmos. Foi decidido que as orientações atualizadas sobre rastreio e tratamento do CCU da OMS seriam desenvolvidas em quatro fases [78].

O resultado da primeira fase foi um grande conjunto de recomendações e declarações de boas práticas sobre rastreio e tratamento, com foco principal na utilização de testes de DNA do HPV e algoritmos clínicos para o rastreio e estratégias de triagem (rastreio primário com testes baseados no DNA do HPV), VIA ou citologia, e testes de triagem

após um rastreio primário positivo, incluindo determinação do genótipo parcial, colposcopia, VIA ou citologia tanto para a população geral de mulheres (ou seja, mulheres que se presume ou se confirma serem seronegativas) como para aquelas que vivem com VIH. Este resultado foi publicado em julho de 2021 [78].

A segunda fase consistiu em desenvolver recomendações para a utilização de testes de mRNA (ácido ribonucleico mensageiro) do HPV como teste de rastreio primário e citologia de dupla coloração como teste de triagem para detetar lesões pré-cancerosas do colo do útero e prevenir o CCU. As recomendações para o uso de testes de mRNA do HPV foram publicadas em dezembro de 2021 [78].

Esta diretriz atual apresenta recomendações para a citologia de dupla coloração como teste de triagem, completando assim a segunda fase. A citologia de dupla coloração pode ser usada como teste de triagem na "abordagem de triagem, triagem e tratamento" para prevenção do CCU. É realizado em lâminas de citologia em base líquida (LBC) (não em exames de Papanicolaou convencionais) para detetar a presença de duas proteínas: p16 e Ki-67. Estas duas proteínas são co expressas em células que foram transformadas por HPV de alto risco. Quando ambas as proteínas são detetadas na(s) mesma(s) célula(s), isso é interpretado como um resultado positivo de citologia de dupla coloração, o que significa que há um risco aumentado de lesões de neoplasia intraepitelial cervical 2/3 (CIN 2/3), também referidas como lesões intraepiteliais escamosas de alto grau (HSIL) [78].

A terceira fase (desenvolvida em 2023) definiu as diretrizes sobre a gestão do CCU invasivo, abordando o diagnóstico, estadiamento e tratamento clínico e cirúrgico. A quarta fase (em curso) refere-se ao desenvolvimento de orientações sobre implementação dos programas de rastreio e tratamento em diferentes contextos de saúde, incluindo estratégias para aumentar a cobertura e adesão [78].

Cada fase teve como objetivo garantir que as recomendações se baseavam nas melhores evidências científicas disponíveis e aprovadas com a meta da OMS de eliminação do CCU como um problema de saúde pública [78].

1.3. - Análise Crítica dos Principais Métodos de Deteção do HPV

O aspeto mais importante a considerar ao decidir qual o método mais adequado para o rastreio e diagnóstico do CCU é que a técnica de deteção do HPV tenha um valor preditivo negativo (VPN) e um valor preditivo positivo (VPP) para CIN 2+ próximo dos 100% de acordo com a classificação de Bethesda [80]. No entanto, uma sensibilidade muito elevada implica geralmente uma perda de especificidade, com inconvenientes importantes para as mulheres, como o sobrediagnóstico, um aumento do número de colposcopias e tratamentos, com impacto socioeconómico [81].

Apesar de existirem muitos métodos disponíveis no mercado para a deteção do HPV em amostras cervicais e a maioria deles cumprir os critérios das diretrizes exigidas [82][83], os únicos testes aprovados pela Food and Drug Administration (FDA) para a deteção do HPV para o rastreio do CCU [84][85] são: o teste Hybrid Capture 2 HPV DNA da Qiagen (Hilden, Alemanha, 2001); Cervista HPV HR test da Hologic (Marlborough, Massachusetts, 2009); Cobas 4800 HPV test da Roche (Basileia, Suíça, 2011); Aptima HPV assay da Gen Probe (San Diego, Califórnia, 2011, adquirido pela Hologic em 2012); BD Onclarity HPV assay da Becton Dickinson (Franklin Lakes, Nova Jersey, 2018) e Cobas 6800/8800 HPV test da Roche (Basileia, Suíça, 2020) [86] (Tabela 4).

Tendo em conta as evidências científicas, é amplamente aceite que o teste para a deteção do HPV deve substituir a citologia como teste inicial no rastreio do CCU, uma vez que é mais sensível, mais facilmente reprodutível, menos subjetivo e, de acordo com os dados publicados, estima-se que previne um maior número de casos de CCU, aproximadamente 32%, proporcionando 60-70% de proteção acrescida contra o CCU do que a citologia [87][88][89].

Outros estudos, como o de Cuzick [90], apoiam esta mudança, uma vez que confirmam que a citologia tem uma sensibilidade muito baixa na deteção de lesões pré-cancerosas quando comparada com os testes HPV (53,0% versus 96,3%), embora seja mais específica (96,3% versus 90,7%). Além disso, outros autores também confirmam que a citologia carece de reprodutibilidade, ou seja, é consideravelmente subjetiva [91].

O aspeto mais importante que tem sido levado em conta para a recomendação de substituir a citologia pelo teste HPV é que a evidência científica demonstra que este último tem um alto VPP para detetar lesões CIN2+, mas especialmente, tem um VPN muito alto, próximo de 100% [92]. Isto significa que, se o teste tiver um resultado

negativo, esta mulher tem uma possibilidade quase nula de lhe serem diagnosticadas lesões pré-cancerosas num período de 5 anos [92].

Em Portugal, são utilizados diferentes testes de deteção do DNA e RNA do HPV, recomendados para o rastreio primário em mulheres com idade superior a 30 anos, onde o teste do HPV demonstra maior sensibilidade na identificação de lesões de alto risco quando comparado com a citologia tradicional.

Table/Tabela 4 - Lista de testes moleculares aprovados pela FDA para deteção de HPV com as principais características.

Test	Year FDA approved	Technique	HPV target	Genotypes detected	Discriminates genotype	Clinical trial	Lesion type/ Sample collected	Sensitivity/ Specifity (%)
QIAGEN digene HC2 High-Risk HPV DNA Test	2001	NA* hybridization + Signal amplification	DNA (L1)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68	No	ASC-US /LSIL Triage Study (ALTS)	HPV HR	94.85/ 88.1
HOLOGIC Cervista HPV HR	2009	NA hybridization + Signal amplification	DNA (L1/E6/E7)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	Yes 16,18)	Cervista HPV HR trial	CIN2+ CIN3+	92.8/ 44.8 100/44.8
ROCHE Cobas HPV for 4800 System	2011	NA amplification	DNA (L1)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	Yes (16,18)	ATHENA trial	CIN2+: PreservCyt® SurePath®	93.8/43.3 94.8/33.6
HOLOGIC Aptima HPV Assay	2011	NA amplification	mRNA (E6/E7)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	Yes 16,18/45)	CLEAR trial	HPV HR CIN2+ CIN3+	92.6/98.5 90.8/55.7 97.7/52.9
BD Onclarity HPV Assay	2018	NA amplification	DNA (E6/E7)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	Yes (16,18, 31, 45,51,52)	Onclarity trial (baseline phase)	CIN2+ CIN3+	85.7/64.1 91.4/62
ROCHE cobas HPV for 6800 /8800 Systems	2020	NA amplification	DNA (L1)	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66,68	Yes (16,18)	IMPACT trial	PreservCyt® SurePath®	93.8/41.7 93.1/43.4

*NA: nucleic acid

Retirado de: Pérez-Gracia, MT, Tarín-Pelló, A., Fernández-Álvarez, S., Más-Comes, C., & Suay-García, B. (2025). Rastreio do Vírus do papiloma Humano e do Cancro do Colo do Útero. Pré-impressões. https://doi.org/10.20944/preprints202501.0982.v1

De seguida, apresentamos um olhar crítico sobre os métodos mais comuns, nomeadamente o **Cobas 4800 HPV Test** e o **Aptima HPV Assay**. Para uma análise crítica dos métodos de deteção do HPV utilizados em Portugal, é fundamental explorar os diferentes testes comercialmente disponíveis, como o teste Cobas, o teste Aptima e outros utilizados para a triagem de infeções por HPV. Esta análise pode incluir a metodologia, especificidade, sensibilidade e as vantagens e limitações de cada método, especialmente no contexto de rastreio do CCU.

1.3.1 - Cobas 4800 HPV Test (Roche Diagnostics)

O teste Cobas é um teste de DNA de HPV que utiliza uma técnica de PCR em tempo real para identificar diretamente o DNA viral. Especificamente, o Cobas 4800 é capaz de diferenciar o HPV 16 e HPV 18, os dois tipos mais associados ao CCU, detetando simultaneamente outros 12 tipos de HPV de alto risco em conjunto [93].

Vantagens:

- **Sensibilidade e especificidade elevadas:** Estudos mostram que o teste Cobas é altamente sensível para a deteção de HPV de alto risco, com especial destaque para o HPV 16 e HPV 18, permitindo uma triagem mais direcionada para o risco de progressão.
- **Capacidade de determinação do genótipo parcial:** Ao identificar especificamente os genótipos 16 e 18, este teste permite que as mulheres com esses tipos de HPV, que têm um risco mais elevado de progressão para o cancro, sejam acompanhadas de forma mais rigorosa [93].

Limitações:

- **Não diferencia todos os tipos de alto risco:** Embora seja possível identificar o HPV 16 e HPV 18 individualmente, os restantes genótipos de alto risco são identificados em conjunto, sem especificidade adicional.
- **Custo e infraestrutura:** Requer equipamentos especializados e profissionais treinados, o que pode limitar a sua acessibilidade em algumas regiões ou em centros com recursos limitados [93].

1.3.2 - Aptima HPV Assay (Hologic)

O teste Aptima é um teste baseado na deteção de RNA mensageiro (mRNA) do HPV de alto risco, utilizando uma técnica de amplificação de sinal (transcription-mediated amplification, TMA). Este método deteta a expressão de mRNA E6/E7, proteínas

envolvidas na oncogénese, o que indica infeções potencialmente mais ativas e clinicamente relevantes [94][95].

Vantagens:

- **Maior especificidade clínica:** A deteção de mRNA em vez de DNA pode ser indicativa de infeções ativas, com maior probabilidade de progressão, o que reduz o número de falsos positivos e o risco de sobre diagnóstico.
- **Redução de procedimentos desnecessários:** A especificidade adicional permite um acompanhamento mais direcionado, evitando procedimentos invasivos em casos de infeção transitória de HPV que, frequentemente, não resultariam em lesões.

Limitações:

- **Sensibilidade ligeiramente inferior ao Cobas para alguns tipos de HPV:** Por se focar na atividade da infeção (via mRNA), alguns estudos indicam que o teste Aptima pode ter uma sensibilidade marginalmente inferior para detetar infeções de baixo risco de progressão, o que é uma vantagem; o ideal será só detetar as infeções com alto risco de progressão [94].
- **Custo:** Como no caso do Cobas, este teste requer equipamentos e infraestrutura específicos, o que pode torná-lo mais caro em comparação com testes de citologia tradicionais[95].

De acordo com as informações disponíveis, o Cobas não é mais barato que o Aptima para a deteção de HPV em Portugal. Uma análise de custos do programa de rastreio do CCU em Portugal mostrou que o uso do teste Aptima HPV resultou em economia de custos em comparação com o Cobas 4800 [97].

1.3.3 - Comparação dos Métodos e Considerações para a prática clínica em Portugal

Existem algumas diferenças na precisão entre o Cobas e o Aptima para a deteção de HPV:

1. O Aptima demonstrou maior sensibilidade e especificidade em comparação com testes baseados em DNA, como o Cobas.
2. Num estudo que avalia a precisão dos métodos Digene HC2 e Aptima para deteção de lesões CIN2 e CIN3:

- Para mulheres com ASC-US inicial, a sensibilidade variou entre 75% e 100% para CIN2 e 93% e 100% para CIN3.
- A especificidade variou entre 20% e 81% para CIN2 e 38% e 81% para CIN3.

3. O Aptima minimiza resultados falso-positivos em comparação com os testes baseados em DNA, como o Cobas.
4. Uma análise de custo-efetividade mostrou que o uso do Aptima HPV resultou em economia de custos em comparação com o Cobas 4800 no programa de rastreio do CCU em Portugal.

Embora ambos os testes sejam altamente precisos, o Aptima parece oferecer algumas vantagens em termos de sensibilidade, especificidade e redução de falsos positivos para a deteção de HPV. No entanto, a escolha entre os dois métodos deve considerar outros fatores além da precisão, como custo, facilidade de uso e disponibilidade local [98].

A escolha do método de rastreio de HPV em Portugal depende de vários fatores, incluindo custo, acessibilidade, especificidade e infraestrutura dos serviços de saúde locais. O teste Cobas é vantajoso para um rastreio mais abrangente e sensível, enquanto o teste Aptima pode ser mais adequado para triagens onde é necessário evitar sobre diagnósticos [99]. A decisão sobre qual teste usar deve considerar as condições da prática clínica, que visam reduzir a incidência do CCU, maximizando a eficácia e a eficiência do rastreio [95]. Os testes Cobas e Aptima oferecem abordagens complementares, uma focada na identificação de infeções de alto risco específicas e outra na avaliação de infeções ativas [95].

Os testes de diagnóstico molecular são mais sensíveis ainda que menos específicos do que a citologia cervical por esfregaço de Papanicolaou, para deteção de lesões pré-malignas principalmente em jovens [94][95].

1.4 - Vantagens e desvantagens da substituição da citologia pelo teste de HPV

A - Vantagens

A substituição da citologia pelo teste de HPV como método primário de rastreio do CCU apresenta várias vantagens, sendo as principais:

1. Maior Sensibilidade na Deteção de Lesões Pré-cancerosas

- O teste de HPV é mais sensível do que a citologia na deteção de lesões de alto grau (lesões intraepiteliais escamosas de alto grau ou HSIL), que têm maior probabilidade de progredir para cancro se não tratadas [102].
- A evidência científica atual indica que a sensibilidade do teste de HPV para lesões de alto grau pode ser superior a 90%, em comparação com cerca de 50% para a citologia. Isso significa que o teste de HPV é mais eficaz para identificar mulheres em risco de desenvolver CCU [96].
- Embora os testes de HPV sejam menos propensos a não detetar casos de CIN 2+ e CIN3+, estes testes levam a mais referenciações desnecessárias. No entanto, um teste de HPV negativo é mais tranquilizador do que um teste citológico negativo, pois o teste citológico tem maior probabilidade de ser falsamente negativo, o que pode levar a atrasos na administração do tratamento adequado [100].

2. Intervalos de Rastreio Mais Longos

- Como o teste de HPV tem um alto VPN, as mulheres que apresentam um resultado negativo podem realizar o rastreio com menos frequência, aumentando o intervalo para cada cinco anos, em vez de três anos, que é o intervalo tradicional para a citologia [102].
- Intervalos de rastreio mais longos reduzem o número de exames e visitas clínicas ao longo da vida das mulheres, resultando em menos intervenções e custos e mantêm a segurança do rastreio [102].

3. Redução dos Resultados Falsos Negativos

- A citologia depende da observação microscópica de células colhidas do colo do útero, o que torna o exame suscetível a erros humanos e à variabilidade na interpretação dos resultados. A qualidade da amostra e a experiência do citopatologista podem influenciar o resultado [96].

- O teste de HPV, sendo um teste molecular, é menos dependente de fatores de subjetividade humana, reduzindo a probabilidade de falsos negativos e aumentando a confiabilidade dos resultados [102].
- O teste de HPV é independente da qualidade da colheita para o rastreio, ao contrário da citologia, em que uma adequada técnica de colheita do material é fundamental para um correto diagnóstico citológico [102].

4. Maior Relevância Clínica para a Identificação de Risco

- A infeção persistente com tipos de HPV de alto risco é necessária para o desenvolvimento do CCU. Assim, o teste de HPV identifica diretamente o fator causal principal deste tipo de cancro, enquanto a citologia apenas deteta alterações celulares, ou seja, doença, enquanto o teste de HPV deteta infeção, quer transitória, quer associada a doença [101].
- A capacidade de identificar infeções específicas de alto risco, como as dos tipos HPV-16 e HPV-18, permite uma melhor estratificação de acordo com o risco, facilitando a orientação para colposcopia e/ou biopsia [103].
- A determinação de genótipo, estendida aos genótipos de HPV de maior risco (HPV 16 e 18) permite uma melhor estratificação do risco (Table/Tabela 5) e a otimização da orientação para colposcopia, bem como a orientação do seguimento, pelo que a sua utilização pode ser considerada no RCCU [104].

Table/Tabela 5 - Risco de HSIL/CIN 3+ em função dos resultados do rastreio do cancro do colo do útero.

	Risco basal (%)	Risco aos 5 anos (%)
HPV negativo	0,0	0,2
HPV 16 positivo	29,9	28,7
HPV 18 positivo	13,2	11,7

Fonte: [105]

A positividade do HPV-hr obtida nos testes de HPV como método de rastreio, se bem que seja útil, é limitada, na medida em que essa identificação do tipo de HPV-hr, é feita através da deteção do vírus nas amostras dos fluidos vaginais, o que nem sempre traduz

com precisão o tipo de HPV presente nas lesões do colo do útero, propriamente dito; a deteção do tipo de HPV através da remoção do DNA viral em material parafinado, será capaz de identificar com precisão o tipo de HPV-hr envolvido no processo de carcinogénese de cada lesão de HSIL do colo do útero, que poderá ser diferente do tipo de HPV detetado nos fluidos do esfregaço cervical; um dos principais obstáculos à utilização da deteção do tipo de HPV em material parafinado na prática clínica é a dificuldade técnica de remoção do DNA viral, o que tem limitado a realização de estudos nesta área. (referido no artigo **High-Risk HPV Detection in Paraffin-Embedded Tissue from Cervical Lesions**)

5. Possibilidade de Auto-colheita

- O teste de HPV pode ser feito com amostras colhidas pelas próprias mulheres, o que não é possível na citologia [107]. Este método tem-se mostrado eficaz e com boa aceitação, especialmente em populações com menor acesso a serviços de saúde, o que aumenta a adesão ao rastreio.
- A auto-colheita pode reduzir barreiras logísticas e culturais para a realização do exame, facilitando a inclusão de mulheres em regiões de difícil acesso e aumentando a cobertura do rastreio [106].

6. Custos Reduzidos a Longo Prazo

- Embora o teste de HPV possa ser inicialmente mais caro que a citologia, a combinação de maior sensibilidade, intervalos de rastreio mais longos e a possibilidade de auto-colheita contribuem para a redução de custos a longo prazo [106].
- A diminuição do número de exames e consultas necessárias ao longo da vida de cada mulher permite uma otimização dos recursos de saúde, tornando o rastreio de HPV uma opção economicamente viável para os sistemas de saúde [99]

B - Desvantagens

Embora o teste de HPV seja uma ferramenta valiosa no rastreio do CCU, a sua utilização como método primário de triagem também apresenta desvantagens e desafios. Destacam-se em seguida as principais desvantagens, com foco no impacto sobre a morbilidade, custos dos programas de rastreio e questões relacionadas com o excesso de tratamentos, especialmente em mulheres jovens.

1. Aumento do Número de Colposcopias

- Com a sensibilidade elevada do teste de HPV, um número significativo de mulheres testadas poderá receber resultados positivos para HPV de alto risco, levando a um aumento do número de colposcopias recomendadas para investigação adicional [101][102].
- Embora a colposcopia seja um procedimento diagnóstico essencial, é invasiva, dispendiosa e pode ser desconfortável e gerar ansiedade nas mulheres, especialmente quando realizadas repetidamente [101][102].
- Este aumento de colposcopias implica uma maior carga de trabalho para os sistemas de saúde, além de aumentar o número de mulheres expostas a procedimentos adicionais que nem sempre resultam em diagnósticos de lesões de alto grau, gerando assim um impacto emocional e psicológico considerável [101].

2. Aumento de Morbilidade por Excesso de Tratamentos

- O teste de HPV é muito sensível, mas essa elevada sensibilidade pode levar a um aumento de casos positivos em mulheres jovens, que geralmente têm maior probabilidade de infeções transitórias por HPV que acabam por ser eliminadas pelo sistema imunitário sem qualquer intervenção [103].
- Em mulheres mais jovens, esse aumento de diagnósticos de HPV pode levar a intervenções invasivas desnecessárias, como biópsias e tratamentos ablativos, elevando o risco de efeitos adversos [106]. Estes incluem desconforto, ansiedade, e, em alguns casos, complicações obstétricas associadas, como aumento da probabilidade de trabalho de parto pré-termo (APPT) em mulheres que foram submetidas a procedimentos no colo do útero [101][106].

3. Sobrecarga de Resultados Positivos e Dificuldade na Gestão Clínica

- A identificação de infeções por HPV-hr sem distinção entre infeções transitórias e persistentes pode levar ao sobrediagnóstico e tratamento excessivo [103].
- O teste de HPV deteta a presença do vírus, mas não indica necessariamente as lesões de alto grau. Esse aumento de resultados positivos cria uma situação de difícil gestão clínica, especialmente em populações mais jovens, onde a maioria das infeções não evolui para lesões significativas [106].

4. Questões Relacionadas com Métodos de Triagem Complementares (e.g., CINtec PLUS)

- Para auxiliar na decisão de seguimento e reduzir o número de intervenções desnecessárias, testes complementares como o **CINtec PLUS** (que identifica a expressão de biomarcadores como p16 e Ki-67, indicando atividade celular anormal) podem ser necessários para melhor estratificar o risco das mulheres com HPV positivo [111][112][114].
- Embora os testes complementares como o CINtec PLUS possam ajudar a reduzir o número de colposcopias desnecessárias, eles adicionam complexidade e custo ao processo de rastreio. A inclusão de um segundo teste aumenta o custo total dos programas de rastreio e exige infraestrutura adicional para análise e interpretação dos resultados [109][110][114][115].
- A dependência de testes complementares também pode complicar os fluxos de trabalho, exigindo uma integração eficaz entre os diferentes métodos de rastreio e decisões de seguimento, o que nem sempre é fácil de implementar em larga escala [108].
- Poderá ser interessante, como exame reflexo, a substituição da citologia pelo CINtec Plus, tal como foi proposto recentemente pela DGS, embora ainda não haja evidência científica consistente sobre a validade dessa metodologia.

Parece não ser ainda consensual, o uso do teste de HPV como método primário de Rastreio do CCU. Até 2018, organizações como a American Society for Colposcopy and Cervical Pathology (ASCCP), a US Preventive Services Task Force (USPSTF) e o American College of Obstetricians and Gynecologists (ACOG) recomendavam o co-teste como o método de rastreio preferencial para mulheres na faixa etária dos 30 aos 65 anos. [97] Em 2018, a USPSTF atualizou as suas recomendações, tendo dado preferência ao rastreio que utiliza apenas um dos métodos individualmente (citologia ou teste de HPV). Por outro lado, o ACOG e a ASCCP mantiveram as suas recomendações de realização de co-teste a cada 5 anos, tendo como opções alternativas a realização de citologia ou teste HPV a cada 3 anos (Tabela 6) [101].

Table/Tabela 6 - Recomendações para rastreio do cancro do colo do útero, ACOG, ASCCP, USPSTF

	ACOG [17]	ASCCP [18]	USPSTF [19]
Somente Pap	A cada 3 anos	A cada 3 anos	A cada 3 anos
Teste de Papanicolau-HPV	A cada 5 anos, idade 30–65	A cada 5 anos, idade 30–65	A cada 5 anos, idade 30–65
Somente HPV de alto risco	A cada 3 anos, idade > 25	A cada 3 anos, idade > 25	A cada 5 anos, idade 30–65

(Fonte [101]. Use of primary high-risk human papillomavirus testing for cervical cancer screening: Interim clinical guidance. J Low Genit Tract Dis. 2015;19(2):91–6.)

1.5 - Estado atual do rastreio em Portugal

Em Portugal, o Rastreio do Cancro do Colo do Útero (RCCU) em 2023 tinha uma cobertura geográfica no território continental e Região Autónoma dos Açores (RAA) de 91% das Unidades Funcionais. Com uma população elegível média de cerca de 562 262 mulheres/ano, a taxa de cobertura populacional foi de 59%. A Taxa de adesão ao rastreio foi de 94%, com um total de 310 976 mulheres rastreadas (301 477 no Continente, 9 499 na RAA). A Região Autónoma da Madeira (RAM) iniciou em 2023 um programa piloto para este rastreio, em 23% dos centros de saúde da região. Do total de mulheres rastreadas, em Portugal continental e RAA, 6,5% (n= 20 206) foram referenciadas para cuidados hospitalares (Tabela 7) [116].

	2021 N (%)	2022 N (%)	2023 N (%)
População Alvo Total	2 654 514	2 838 286	3 080 239
População Elegível	2 628 857	2 768 307	2 873 353
População Excluída	25 657	69 979	140 134
População Elegível no Ano	525 771	553 661	562 262
Convidadas (Taxa de Cobertura Populacional)	265 988 (51%)	353 057 (64%)	332 644 (59%)
Rastreadas (Taxa de Adesão)	251 224 (94%)	330 859 (94%)	310 976 (94%)
Testes Primários Positivos	27 494 (11%)	38 353 (12%)	41 973 (14%)
Critérios Referenciação Hospitalar	16 538	16 559	20 206

Fonte: ARS e COA, 2022 e 2023 e NCR/DE-SNS e COA 2024.

Table/Tabela 7 - Sumário do Desempenho do Rastreio do Cancro do Colo do Útero - Portugal Continental e RAA | 2021 – 2023

No entanto, e apesar da introdução de programas de rastreio organizados e de base populacional, continua a verificar-se a existência de diferenças nas taxas de adesão ao rastreio do CCU entre as várias zonas do território. Foi também descrita a existência de desigualdades na realização do rastreio do CCU no nosso país antes da introdução destes programas, sendo que estas desigualdades estavam associadas tanto a características socioeconómicas e comportamentais da população, como a fatores relacionados com a utilização dos serviços de saúde [117].

A não frequência de mulheres em idade de rastreio nos programas de rastreio do CCU é de cerca de 22% [116] em Portugal; por outro lado, de acordo com um estudo que incluiu rastreios organizados e oportunistas, mostrou que a não frequência é de cerca de 13,2% (a prevalência ponderada da não adesão foi de 13,2% [intervalo de confiança (IC) de 95%: 12,0-14,0] [119].

O rastreio do CCU tem como população alvo mulheres, assintomáticas, com idade compreendida entre os 25 e os 60 anos, convidadas de 5 em 5 anos para a colheita de exsudado cervico-vaginal para deteção de DNA do vírus HPV dos serotipos de alto risco, como teste de rastreio primário. Este programa de rastreio permite a deteção e tratamento de lesões pré-malignas, bem como o diagnóstico e tratamento precoce do CCU com o consequente impacto na redução da incidência e da mortalidade, respetivamente [116].

De acordo com o resultado do teste de rastreio primário é realizado o encaminhamento da utente [112]:

• Pesquisa de DNA viral do HPV:

- Teste negativo: realização de novo rastreio no prazo de cinco anos.

- Teste positivo com identificação de subtipo 16 ou 18: referenciação para consulta hospitalar de Ginecologia, área de patologia cervical, para diagnóstico, tratamento ou acompanhamento;

- Teste positivo com identificação de subtipo não 16 ou não 18: realização de citologia reflexa no material biológico previamente colhido e gestão em função do resultado:

- Células atípicas escamosas de significado indeterminado (ASC-US), de alto grau (ASC-H); Células atípicas glandulares (AGC); Lesão epitelial de baixo grau (LSIL) ou alto grau (HSIL): referenciação para consulta hospitalar de Ginecologia, área de patologia cervical, para diagnóstico, tratamento ou acompanhamento;

- Citologia negativa (NILM): realização de novo rastreio no prazo de um ano (no caso de uma citologia cervico-vaginal realizada no contexto de teste HPV positivo com identificação de subtipo não 16 ou não 18).

Em 2023 este rastreio esteve implementado em todos os ACeS de Portugal Continental e RAA, tendo sido convidadas 332 644 mulheres (correspondendo a uma taxa de cobertura populacional de 59%) das quais 310 976 foram rastreadas (taxa de adesão de 94%). Foram identificadas 41 973 mulheres com teste primário positivo das quais 20 206 apresentavam critérios de referenciação para o hospital para diagnóstico / tratamento / acompanhamento [116].

As novas recomendações europeias consideram a idade para realização do rastreio dos 30 aos 65 anos de idade, a cada 5 anos, considerando a adaptação das idades e dos intervalos ao risco individual com base no historial de vacinação contra o HPV de cada pessoa, e referem a possibilidade de oferecer kits para auto-colheita. Estas recomendações estão a ser consideradas para implementação em Portugal [120].

Nas Tabela 8, Tabela 9, e Tabela 10, descreve-se o desempenho do programa nacional de rastreio do CCU em Portugal Continental e RAA.

	População Elegível Anual			Nº Utentes Convidados			Cobertura Populacional		
	2021	2022	2023	2021	2022	2023	2021	2022	2023
Norte	192 020	213 914	211 296	127 568	159 338	154 260	66%	74%	73%
Centro	86 044	75 048	74 256	59 749	69 167	47 110	69%	92%	63%
LVT	186 895	199 599	211 743	49 676	82 355	88 630	27%	41%	42%
Alentejo	23 132	23 252	26 001	11 171	19 860	19 983	48%	85%	77%
Algarve	24 844	28 766	25 616	7 429	10 824	11 049	30%	38%	43%
RAA	12 835	13 082	13 350	10 395	11 513	11 689	81%	88%	87%
Total	525 770	553 661	562 262	265 988	353 057	332 644	51%	64%	59%

Fonte: ARS e COA, 2022 e 2023 e NCR/DE-SNS e COA 2024.

Table/Tabela 8 - Comparação da População Elegível e Cobertura Populacional – Rastreio do CCU - Portugal Continental e RAA | 2021 – 2023

	Nº Utentes Convidados			Nº Utentes Rastreados			Taxa Adesão		
	2021	2023	2023	2021	2022	2023	2021	2022	2023
Norte	127 568	159 338	154 260	119 966	147 799	142 919	94%	93%	93%
Centro	59 749	69 167	47 110	58 190	66 761	45 150	97%	97%	96%
LVT	49 676	82 355	88 630	48 013	79 975	86 404	97%	97%	98%
Alentejo	11 350	19 860	19 983	11 171	16 817	16 240	98%	85%	81%
Algarve	7 429	10 824	11 049	7 228	10 537	10 764	97%	97%	97%
RAA	10 395	11 513	11 689	7676	8 970	9 499	74%	78%	81%
Total	266 167	353 057	332 644	252 244	330 859	310 976	95%	94%	94%

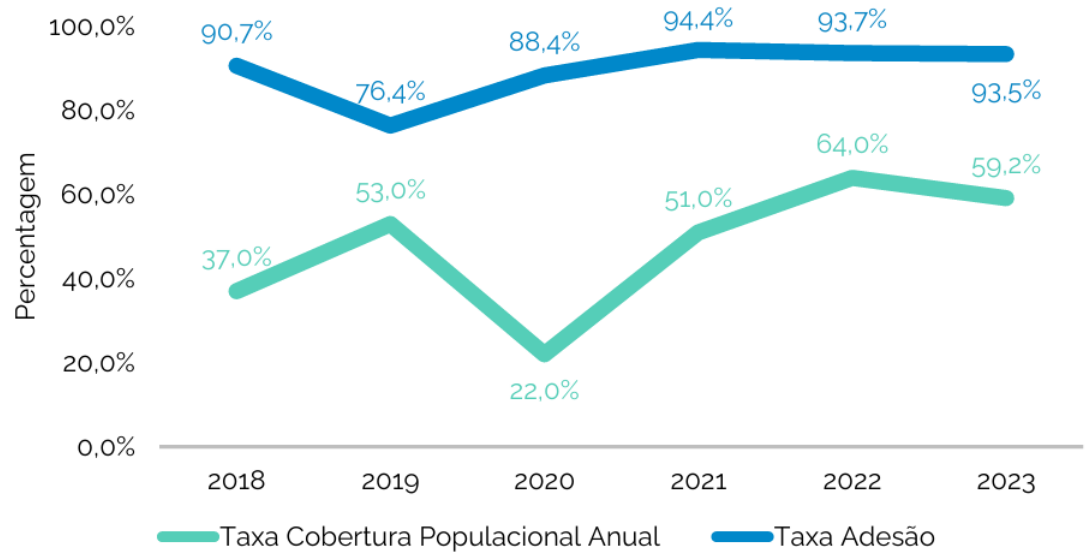
Table/Tabela 9 - Comparação Nº Convidados, Nº Rastreados e Taxas de Adesão – Rastreio do CCU – Portugal Continental e RAA, entre 2021 – 2023. Fonte: ARS e COA, 2022 e 2023 e NCR/DE-SNS e COA 2024.

	2020	2021	2022
Taxa Cobertura Geográfica por ACeS	100%	100%	100%
Taxa Cobertura Geográfica por UF	86%	89%	91%
Taxa Cobertura Populacional Anual	53%	64%	59%
Taxa Rastreio Populacional Anual	40%	60%	55%

Table/Tabela 10 - Evolução das Taxas de Cobertura Geográfica, Populacional e de Rastreio – Rastreio do CCU – Portugal Continental e RAA, entre 2020 – 2022. Fonte: ARS e COA, 2022 e 2023 e NCR/DE-SNS e COA 2024.

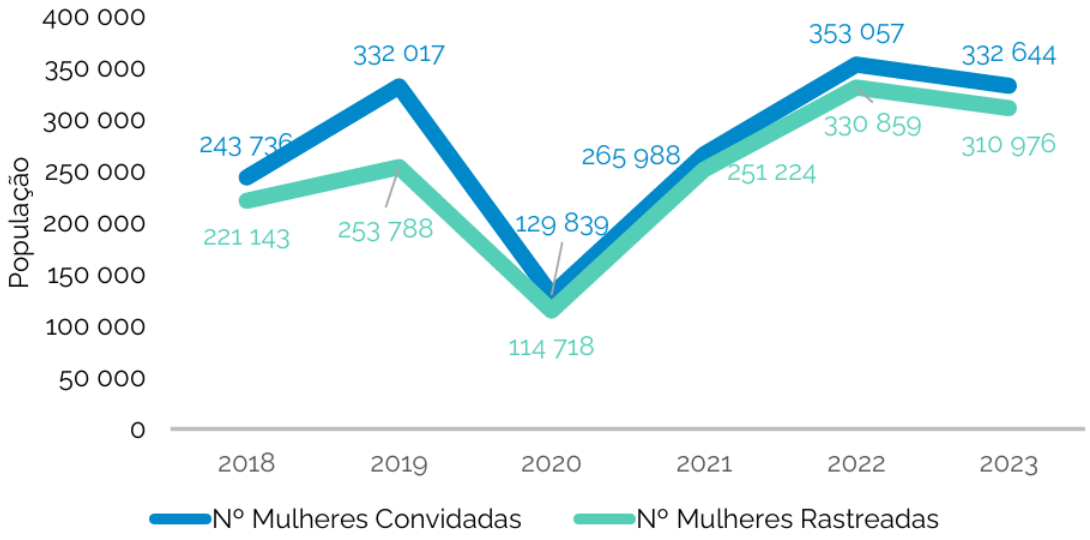
Nos gráficos 1 e 2 expõe-se a evolução histórica do RCCU entre 2018 e 2023.

Gráfico 1 - Taxa de Cobertura Populacional Anual e Taxa de Adesão - Rastreio do CCU - Portugal Continental e RAA | 2018 – 2023



Fonte: ARS e COA, 2019 a 2023 e NCR/DE-SNS e COA 2024.

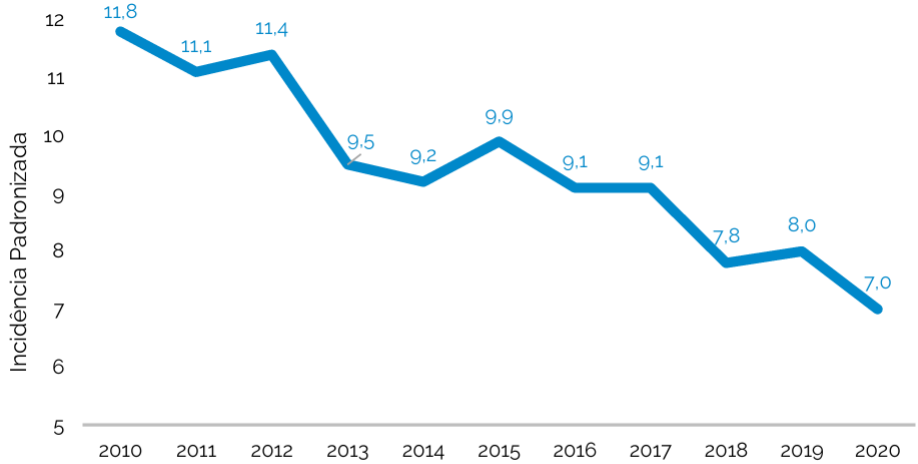
Gráfico 2 - Evolução do Nº Convidadas e Rastreadas – Rastreio do CCU - Portugal Continental e RAA | 2018 – 2023



Fonte: ARS e COA, 2019 a 2023 e NCR/DE-SNS e COA 2024.

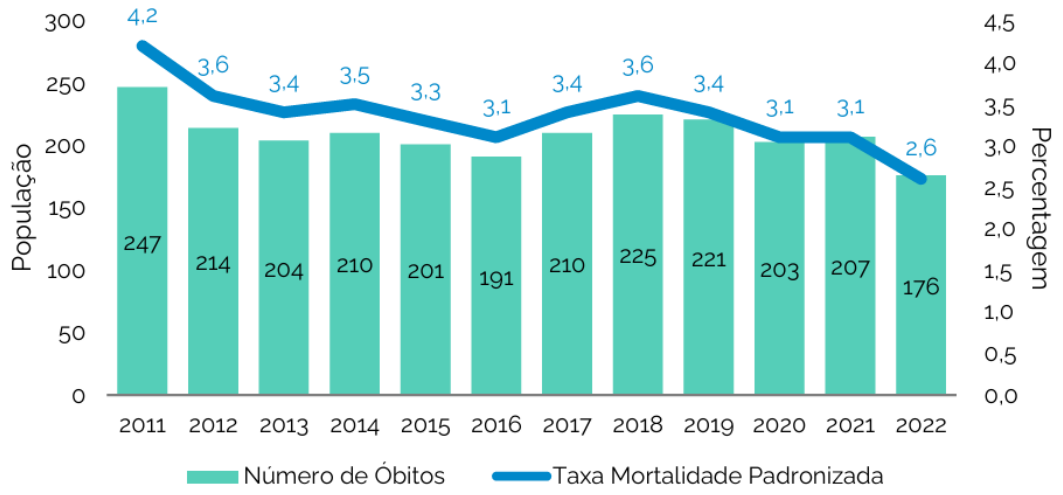
Nos últimos anos, a taxa de incidência padronizada tem vindo a descer e a taxa de mortalidade padronizada e o número de óbitos estão estabilizados (Gráficos 3 e 4).

Gráfico 3 - Taxa de Incidência Padronizada do CCU | 2010 – 2020



Fonte: De 2011 a 2017: dados de publicações do Registo Oncológico Nacional elaboradas pelos ROR-Sul, ROR Centro e RORENO. De 2018 a 2021: dados da Plataforma RON. Considera-se a existência de uma quebra de série entre 2017 e 2018. Incidência Padronizada ajustada pela idade à população europeia 1976.

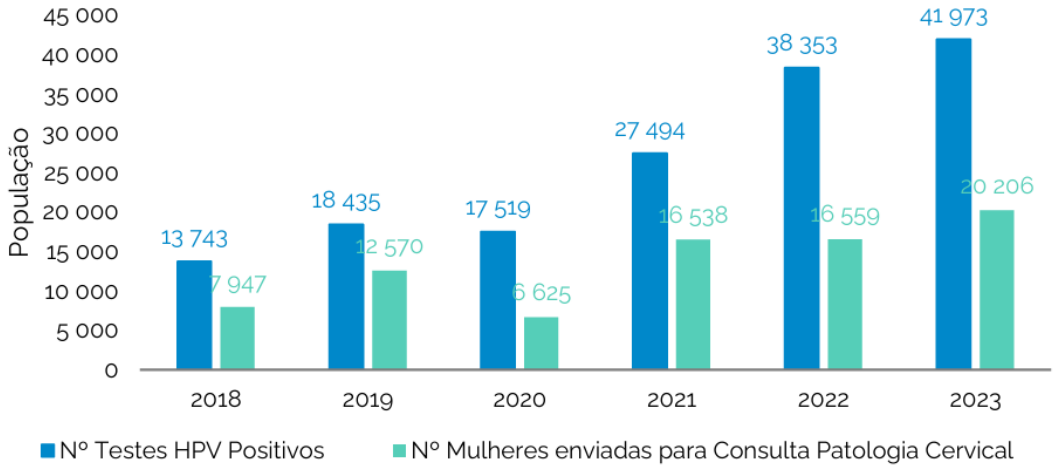
Gráfico 4 - Taxa de Mortalidade Padronizada e Nº Óbitos do CCU | 2011 – 2022



Fonte: Óbitos por causas de morte, INE (2024). Taxas padronizadas calculadas pela DSIA/DGS, com base na população padrão europeia (versão 2013) definida pelo EUROSTAT e utilizando o método direto de padronização e grupos etários quinquenais. Tumor maligno do colo do útero Código C53 da CID10. Taxas expressas em número de óbitos por 100 000 habitantes. Os valores das taxas de mortalidade para o ano 2020 foram revistos na sequência da divulgação pelo INE das Estimativas Definitivas de População Residente – valores revistos em março de 2023 (revisão regular geral), em função dos resultados definitivos dos Censos 2021.

A atividade deste rastreio em termos de mulheres convidadas e rastreadas baixou ligeiramente em 2023 em comparação com o ano anterior, tal como observado no Gráfico 5.

Gráfico 5 - Evolução do Nº Testes HPV Positivos e Nº Mulheres enviadas para Consulta de Patologia Cervical – Rastreio do CCU| 2018 – 2023



Fonte: NCR/DE-SNS e COA 2024.

A taxa de mortalidade por CCU em Portugal, situa-se nos níveis muitos altos de IDH.

Em Portugal, em 2020, foi determinada uma incidência e mortalidade padronizada para a idade de 10,7 e 3,2/100 000 mulheres/ano, respetivamente [10]. Provavelmente, a taxa de cobertura do rastreio é suficientemente baixa para justificar tal número.

Em contraste com o rastreio populacional organizado, o rastreio oportunista, ainda muito usado em Portugal [119], depende da iniciativa da mulher individual e/ou do seu médico. Este tipo de rastreio resulta frequentemente numa cobertura elevada apenas em certos grupos da população, que são rastreados frequentemente, enquanto outros, normalmente com um estatuto socioeconómico mais baixo, apresentam uma cobertura mais baixa. Esta situação resulta numa cobertura desigual com qualidade heterogénea, eficácia limitada e custo-eficácia reduzida, bem como dificuldade na monitorização da população [92].

Portugal implementou em 2017 um programa nacional de rastreio baseado no teste primário de HPV para mulheres com idades dos 25 aos 60 anos. No entanto, este programa não está totalmente implementado em todas as regiões, o que contribui para as atuais dificuldades que o rastreio organizado atravessa, com apenas 64 % das mulheres a participarem no Rastreio em 2022, pelo que é lícito afirmar que o rastreio oportunístico (ou de conveniência) tem um papel importante na prevenção do CCU.

Significa que muitas mulheres em Portugal, como em outros países, farão prevenção secundária do CCU de forma oportunística, pelo que será conveniente elaborar orientações para este tipo de rastreio. Visto que estas mulheres, muito provavelmente suportam os custos, há que considerar uma atuação clínica que leve isso em conta.

Os estudos efetuados, de que resultaram os artigos científicos publicados, sobre a experiência do programa de rastreio da ULSCB (das mulheres que aderiram ao programa de rastreio oportunístico na Consulta de Ginecologia do Hospital da Covilhã) são a base do contributo que se pretende deixar para a melhoria da eficácia da prevenção secundária do CCU em Portugal.

Neste sentido, entre muitas questões que se podem colocar, questionámo-nos por:

1º) valerá a pena fazer um co-teste, mesmo nas mulheres com teste de HPV 16 e ou 18 positivo?

2º) Que fazer a mulheres com testes de HPV não 16 ou 18 positivos repetidos com citologia sem alterações ou alterações minor?

3º) Será possível encontrar uma técnica prática e adequada para a determinação do genótipo do HPV em material parafinado, que possa no futuro vir a orientar a conduta terapêutica das displasias cervicais?

Visto que a maioria da prevenção secundária é efetuada por médicos especialistas em Ginecologia, com experiência e capacidade de valorização macroscópica do colo do útero, outras questões se tornam pertinentes, tais como: 1º) Quando começar a prevenção; 2º) quando terminar; qual a conduta em mulheres vacinadas; 3º) que periodicidade? Entre outras.

O programa de rastreio em Portugal tem sido ajustado, no que se refere à idade de início e de término, para refletir os avanços na vacinação e nas evidências epidemiológicas:

- O rastreio, anteriormente iniciado aos 25 anos, passa a começar aos 30 anos, devido à elevada taxa de vacinação nas faixas etárias mais jovens .
- A idade-limite para o rastreio foi alargada de 60 para 69 anos, considerando que 19% dos casos de cancro do colo do útero diagnosticados em 2018 ocorreram em mulheres entre os 60 e 70 anos .

Table/Tabela 11 - Resultados dos testes de rastreio e Referenciação para a Consulta de Patologia Cervical (retirado da NORMA N.º 09/2024 de 17/10/2024)

Resultado do Teste Primário *	Resultado da citologia com dupla marcação imunoquímica p16/Ki67 §	Unidade de Patologia Cervical ǂ
Negativo	Não aplicável	Não aplicável
Positivo		
HPV 16 ou 18	Não aplicável	Nível 1 (30 dias seguidos)
HPV não 16 ou 18	Positivo	Nível 1 (30 dias seguidos)
	Negativo	Não aplicável
Inconclusivo	Não aplicável	Não aplicável

*

De acordo com metodologia laboratorial assente na pesquisa de ácidos nucleicos dos vírus HPV, que inclua os genótipos 16, 18 e os genótipos de alto risco não 16 ou 18 (nomeadamente: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) utilizando um dispositivo médico para diagnóstico in vitro (DIV) com marcação CE.

§ A avaliação imunoquímica para pesquisa das proteínas p16/Ki67 deve utilizar um teste diagnóstico in vitro (DIV) com marcação CE.

ǂ O prazo para a realização de primeira consulta de acordo com a classificação definida para efeitos de determinação dos tempos máximos de resposta garantida no SNS para Neoplasias Malignas, confirmadas ou suspeitas (Portaria n.º 153/2017, de 4 de maio)

Capítulo 2 – Objetivos

O **objetivo principal** que esteve na base desta tese, foi avaliar o interesse em utilizar o teste de HPV-hr como método primário de rastreio do cancro do colo do útero, utilizando o protocolo de rastreio de conveniência do Centro Hospitalar Universitário da Cova da Beira, para retirar os ensinamentos que possam contribuir para uma melhor rentabilização do Rastreio Oportunístico/Conveniência do CCU, efetuado no âmbito das Consultas Médicas, sobretudo quanto a:

1º) Avaliar o risco de HSIL em mulheres com teste de HPV-hr (não 16 e/ou 18) positivos repetidos e com citologia NILM;

2º) Verificar se é válido dispensar a realização da citologia nos casos de teste de HPV-hr positivo para o HPV 16 e 18, com referenciação imediata à colposcopia;

3º) Desenvolver uma técnica laboratorial, que permita uma deteção adequada e pouco dispendiosa, para a determinação do genótipo do HPV em material parafinado, possibilitando a elaboração de um prognóstico de morbilidade.

Capítulo 3 – Material e métodos

Na procura das respostas para os objetivos da nossa investigação, foram realizados dois estudos clínicos, transversais e retrospetivos, tendo como base de dados as mulheres com mais de 25 anos que frequentaram as consultas de Ginecologia do CHUCB, entre agosto de 2012 e agosto de 2017, para o primeiro estudo e entre agosto de 2012 e junho de 2021, para o segundo. Foi também critério de inclusão as mulheres não terem tido algum tipo de rastreio e/ou follow-up nos últimos dois anos. Foram critérios gerais de exclusão as mulheres terem já sido submetidas a histerectomia total, assim como terem tido algum tipo de rastreio e/ou follow-up nos últimos dois anos.

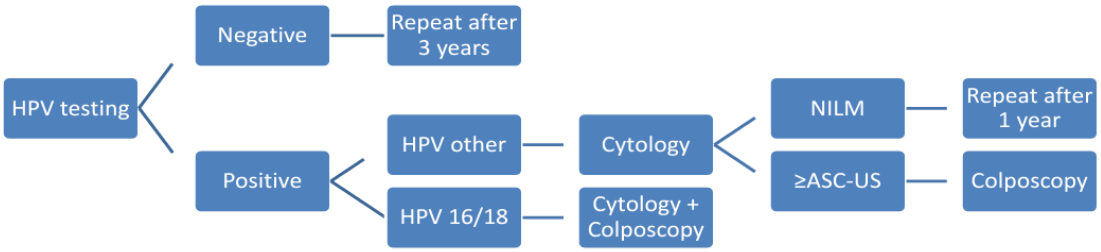
Nestes dois estudos, foi utilizado o protocolo de rastreio de rotina do CCU Oportunístico/Conveniência em vigor no CHUCB, utilizando como método primário de triagem, o teste Cobas®4800 HPV, que deteta HPV 16, HPV 18 e outros tipos de HPV-hr (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68), utilizando o meio líquido Surepath®.

Os estudos foram revistos e aprovados pela Comissão de Ética da Universidade da Beira Interior e realizados de acordo com as orientações e regulamentos relevantes. A área de influência do CHUCB, inclui os municípios da Covilhã, Fundão, Belmonte e Penamacor, e o centro hospitalar presta cuidados a uma população de aproximadamente 90.000 habitantes, pelo que a dimensão da amostra foi limitada.

Todos os testes de HPV foram realizados no Laboratório do Serviço de Patologia Clínica do CHUCB, enquanto os exames citológicos e histológicos foram realizados no Departamento de Anatomia Patológica do CHUCB.

O programa de rastreio do CCU do CHUCB foi concebido e implementado pela Unidade de Colposcopia do CHUCB, onde foram realizados todos os exames colposcópicos. De acordo com o algoritmo de rastreio do CHUCB, apresentado na Figure/Figura 7, um teste de HPV negativo deveria ser repetido após 3 anos; um teste de HPV positivo para os genótipos 16 ou 18 é seguido de citologia reflexa e encaminhamento para colposcopia; um teste de HPV positivo para outros tipos de HPV-hr é seguido de citologia reflexa, e se a citologia reflexa mostrar NILM, o teste deve ser repetido após 1 ano; qualquer outro achado citológico requer encaminhamento para colposcopia. Após um segundo teste de HPV positivo consecutivo, a mulher é seguida na Unidade de Colposcopia por pelo menos

3 anos, independentemente dos resultados subsequentes do teste de HPV e dos achados citológicos.



Figure/Figura 7 - Fluxograma do Rastreio do Cancro do Colo do Útero Implementado no Centro Hospitalar Universitário da Cova da Beira (CHUCB)

No primeiro estudo, dos 6.003 testes de HPV-hr realizados em 5.227 mulheres que realizaram exames de rotina no CHUCB de agosto de 2012 a agosto de 2017, 765 (14,6%) mulheres que tiveram um teste de HPV positivo foram selecionadas para o nosso estudo. Destas, avaliamos 141 mulheres que não tinham histórico de tratamento para neoplasia intraepitelial cervical e apresentavam achados citológicos satisfatórios, classificadas como NILM ou lesões citológicas minor (ASC-US/LSIL), colposcopia normal e/ou sem displasia na biópsia, e que realizaram consulta de seguimento no Serviço de Colposcopia do CHUCB. Na Consulta de Colposcopia, na presença de resultados colposcópicos de grau 1 ou 2, ou sinais de invasão, era realizada biópsia do colo. Quando a zona de transformação (ZT) era classificada como tipo 3 (junção escamocolunar não totalmente visível), era realizada por rotina, curetagem endocervical.

Foi realizada análise estatística descritiva dos dados, utilizando o software aplicativo IBM SPSS, versão 26 (SPSS Inc., Chicago, IL). Em todos os casos, analisámos a idade da paciente, os resultados dos testes de HPV-hr (tipo 16, 18 e outros) e os resultados citológicos e achados histológicos das biópsias orientadas por colposcopia.

No segundo estudo clínico, foram incluídas 6.376 mulheres que foram submetidas ao rastreio de rotina do CCU no CHUCB durante os 9 anos acima mencionados (agosto de 2012 - junho de 2021), às quais foram realizados 8.022 testes de HPV-hr, tendo as 339 (5,3%) mulheres que testaram positivo pela primeira vez para os genótipos 16 e/ou 18 do HPV e submetidos à citologia reflexa, sido selecionados para o nosso estudo. Em todos esses casos, analisámos os resultados do teste de HPV-hr da paciente (tipo 16 e/ou 18) e os achados citológicos e histológicos das biópsias obtidas por colposcopia

(imediatamente e após 3 anos). Para análise dos dados, como houve apenas dois casos de células escamosas atípicas (ASCH), estes foram incluídos na citologia HSIL+.

Os dados foram inseridos e analisados utilizando SPSS Statistics for Windows, versão 21.0 (Armonk, NY: IBM Corporation). Proporções, médias aritméticas, medianas e desvios padrão (DP) foram utilizadas como estatísticas resumidas.

Para explorar a possibilidade de desenvolver uma técnica, adequada e pouco dispendiosa, que permita a determinação do genótipo do HPV em material parafinado, que nos oriente para um prognóstico de morbilidade, e risco de progressão das lesões de HSIL, realizámos um estudo laboratorial retrospetivo, utilizando amostras de tecido embebidas em parafina, de 45 mulheres previamente submetidas à excisão de lesão cervical escamosa de alto grau na Unidade Local de Saúde da Cova da Beira. A colheita de amostras ocorreu no Departamento da Criança e da Mulher, na unidade de Oncologia Ginecológica da ULSCB, Covilhã, Portugal. Este estudo foi aprovado pela Comissão de Ética da Universidade da Beira Interior com o código CE-UBI-Pj-2017-027.

Depois de processadas lâminas de tecido embebidas em parafina de 3 μm de espessura, estas são coradas com hematoxilina e eosina. Estas lâminas foram de seguida avaliadas por dois patologistas independentes, para confirmar a presença de HSIL. Após a confirmação de HSIL, foi realizada a extração de DNA.

O processo de preparação das lâminas para extração de DNA envolve várias etapas para evitar contaminação cruzada e garantir a pureza do DNA. O primeiro passo na otimização da extração de DNA foi obter a espessura correta do tecido; assim, lâminas de biópsia embebidas em parafina de 10 μm foram obtidas para cada caso confirmado de HSIL.

Após o processo de corte, as lâminas foram submersas em xileno até que a lâmina estivesse completamente coberta. A incubação foi realizada usando um novo tubo falcon estéril de 50 ml para cada lâmina, a fim de evitar contaminação cruzada. Esta etapa é crucial para a desparafinização porque a parafina, que incorpora e preserva o tecido, deve ser completamente dissolvida, permitindo a exposição do tecido subjacente e a subsequente extração de seu DNA.

As lâminas foram então transferidas individualmente para um novo tubo falcon estéril de 50 ml, e etanol absoluto foi usado até que a lâmina estivesse completamente

submersa. O período de incubação foi de 5 min em temperatura ambiente. Depois disso, as lâminas foram deixadas para secar em temperatura ambiente por 5 a 10 min.

O passo seguinte envolveu a recuperação física do tecido da lâmina. Foi usado um bisturi estéril novo para cada amostra; o tecido foi cuidadosamente raspado da lâmina para um microtubo de 1,5 ml. Este é um processo delicado que requer precisão para garantir que o tecido seja recolhido com sucesso, sem contaminação. Nenhum fluxo deve estar presente para evitar a perda do tecido raspado.

A extração do DNA genómico foi realizada usando o QIAamp DNA FFPE Tissue Kit (Qiagen, Germantown, MD, EUA) de acordo com as instruções do fabricante. O protocolo fornecido pelo fabricante foi seguido meticulosamente, e pontas de pipeta filtradas foram usadas. Todo o processo de extração de DNA foi realizado numa estação de trabalho dedicada para manter um ambiente livre de contaminação. Após a extração, as amostras foram armazenadas a -20 °C.

Para a determinação do genótipo do HPV-hr, foi utilizado o kit Anyplex™ II HPV HR Detection, Catálogo Nr. HP7E00X, (Seegene ®, Seul, República da Coreia, adquirido para Werfen, Portugal, seguindo as instruções do fabricante). Foi realizada uma PCR multiplex em tempo real (CFX96 PCR da Bio-Rad, Hercules, CA, EUA). O kit permite a determinação simultânea do genótipo de 14 tipos de HPV, incluindo 12 tipos de alto risco identificados como carcinógenos do Grupo 1 (HPV-16/18/31/33/35/39/45/51/52/56/58/59). Além disso, abrange o HPV-66, classificado no Grupo 2B como possivelmente carcinogénico, e o HPV-68, classificado no Grupo 2A como provavelmente carcinogénico. O kit Anyplex™ II HPV HR Detection fornece um controle interno para cada amostra e controles positivos e negativos para cada placa para a reação de PCR em tempo real. O sistema de controle meticuloso é essencial para garantir a precisão e a confiabilidade dos resultados de PCR, oferecendo uma camada adicional de validação ao processo de determinação do genótipo. Ao incluir esses controlos, o kit minimiza efetivamente o potencial de resultados falso-positivos ou falso-negativos, proporcionando assim um maior grau de confiança.

Para evitar contaminação cruzada, a mistura de PCR foi preparada dentro de um gabinete de PCR UV. Após esta etapa, a adição de DNA foi realizada num gabinete de fluxo laminar vertical. Este gabinete garante um fluxo de ar estéril, salvaguardando a integridade das amostras e a precisão do processo de determinação do genótipo.

Para a determinação do genótipo, também foram utilizadas pontas de pipetas filtradas, também como pipetas dedicadas, para evitar contaminação cruzada.

Os dados de PCR foram analisados usando o software Seegene Viewer™ (Seegene ®), versão 3, que é projetado especificamente para interpretar dados gerados por PCR multiplex em tempo real. Os controlos internos do kit e o algoritmo do software permitiram-nos confirmar a amplificação do DNA e o(s) tipo(s) de HPV-hr presentes em cada HSIL.

Capítulo 4 – Resultados

Os três estudos desenvolvidos no âmbito desta tese, de que resultaram os artigos científicos publicados, visam de forma integrada, avaliar o contributo de um rastreio oportunista para a melhoria da prevenção do cancro do colo do útero.

Cada estudo aborda uma dimensão distinta, mas complementar do processo de rastreio, desde a identificação inicial de infeções por HPV até à confirmação laboratorial da sua relevância clínica.

O primeiro estudo, "Repeated Positive Cervical HPV Testing and Absent or Minor Cytology Abnormality at Pap Smear. What is the Next Step?", analisa a problemática da persistência de infeções por HPV em mulheres com citologia normal ou com alterações ligeiras. Os resultados obtidos evidenciam a existência de um número significativo de casos de HSIL em mulheres com positividade persistente para HPV, mesmo na ausência de alterações citológicas relevantes, o que aponta para a vantagem da colposcopia nessas situações.

O segundo estudo, "HPV testing for cervical cancer screening: Should reflex cytology be performed after a positive test for HPV 16 and 18?", assume um papel central na avaliação de estratégias de gestão após um teste positivo para HPV de alto risco. Através da análise da eficácia da citologia reflexa como método de triagem secundária, este estudo discute se esta abordagem é suficiente para garantir a deteção precoce de lesões clinicamente significativas ou se, pelo contrário, se justifica o encaminhamento direto para colposcopia nos casos positivos para HPV 16 e 18. Os resultados apontam que, nestes casos, a citologia concomitante (co-teste) não trouxe benefícios adicionais para o diagnóstico das lesões de HSIL, pelo devem ser dispensadas, evitando assim gastos desnecessários para as mulheres que fazem rastreio oportunístico.

O terceiro estudo, "High-Risk HPV Detection in Paraffin-Embedded Tissue from Cervical Lesions", fornece uma técnica de fácil execução e baixo custo para a deteção de HPV de alto risco em amostras de tecido fixado em parafina. Esta componente laboratorial permite detetar a presença de HPV 16 e 18 em lesões de HSIL histologicamente diagnosticadas, confirmando que na carcinogénese da lesão em causa esteve envolvido o HPV 16/18. Os dados obtidos podem permitir no futuro uma melhor gestão das mulheres com o diagnóstico de HSIL do colo do útero.

Em conjunto, os três estudos demonstram que, mesmo num contexto de rastreio oportunista, é possível melhorar a prevenção do cancro do colo do útero através da implementação de estratégias que combinem a deteção precoce do HPV de alto risco com a gestão criteriosa dos casos positivos (em especial dos genótipos 16 e 18). Estes contributos podem ser cruciais para a rentabilização das atitudes clínicas a adotar no rastreio oportunístico do cancro do colo do útero.

4.1 - Repeated Positive Cervical HPV Testing and Absent or Minor Cytology Abnormality at Pap Smear. What is the Next Step?

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4.1.1 - Abstract

Background: Human papillomavirus (HPV) screening has significantly reduced cervical cancer (CC) mortality. Women who consecutively test positive for high-risk HPV without and minor changes on reflex cytology (atypical squamous cells of undetermined significance [ASC-US] or low-grade squamous intraepithelial lesion [LSIL]) or dysplasia on cervical colposcopy-oriented biopsy are always referred to colposcopy. The aim of the present study was to assess whether this guidance is appropriate for COBAS HPV testing with reflex cytology.

Methods: *A* cross-sectional, retrospective study was carried out in 5227 women who underwent routine CC screening over a period of five years (2012-2017). All HPV tests were performed using Cobas®4800 HPV. The study included women attending gynecology appointments whose first HPV test was positive and who had any type of follow-up. Patients' HPV test results as well as cytology and biopsy findings obtained during the abovementioned period were analyzed. A descriptive and comparative statistical study was conducted using this data.

Results: A total of 765 out of 6003 HPV tests performed in 5227 women were positive, and 141 women who had a positive HPV test (with negative for intraepithelial lesion or malignancy [NILM] or inflammation, or ASC-US and LSIL cytology, but no lesions on colposcopy, or absence of dysplasia on histology) repeated the HPV test at least once. Of these 141 women, 6 were diagnosed with high-grade squamous intraepithelial lesion (HSIL) during the follow-up period. All cases of HSIL were diagnosed after the second HPV test.

Conclusion*:* This study shows that, at cervical cancer screening, all women testing positive for HPV regardless of Pap smear result should be referred to colposcopy.

Keywords: Cervical cancer, HPV testing, cervical cancer screening, HSIL, CIN

Abbreviations

ASCCP – American Society for Colposcopy and Cervical Pathology; ASC-H – atypical squamous cells; ASC-US – atypical squamous cells of undetermined significance; CC – cervical cancer; CHUCB – Cova da Beira University Hospital Center; CIN – cervical intraepithelial neoplasia; Co-testing: Concomitant HPV test and cytology; HPV – human papillomavirus; HSIL – high-grade squamous intraepithelial lesion; KPNC – Kaiser

Permanent Northern California; LSIL – low-grade squamous intraepithelial lesion; NILM – negative for intraepithelial lesion or malignancy; SPSS – Statistical Package for the Social Sciences.

Resumo

Antecedentes: O rastreio do papilomavírus humano (HPV) reduziu significativamente a mortalidade por cancro do colo do útero (CCU). Mulheres que apresentam resultados consecutivos positivos para HPV de alto risco sem alterações ou com alterações menores na citologia reflexa (células escamosas atípicas de significado indeterminado [ASC-US] ou lesão intraepitelial escamosa de baixo grau [LSIL]) ou displasia na biópsia cervical orientada por colposcopia, são sempre encaminhados para colposcopia. O objetivo do presente estudo foi avaliar se esta orientação é apropriada para a utilização do teste COBAS HPV com citologia reflexa.

Métodos: Foi realizado um estudo transversal e retrospetivo em 5.227 mulheres submetidas ao rastreio de rotina do CCU durante um período de cinco anos (2012-2017). Todos os testes de HPV foram realizados utilizando Cobas®4800 HPV. Foram incluídas no estudo mulheres que compareceram às consultas de ginecologia cujo primeiro teste de HPV foi positivo e que tiveram algum tipo de acompanhamento. Foram analisados os resultados dos testes de HPV dos pacientes, bem como os achados de citologia e biópsia obtidos durante o período acima mencionado. Foi realizado um estudo estatístico descritivo e comparativo com esses dados.

Resultados: Um total de 765 dos 6.003 testes de HPV realizados em 5.227 mulheres foram positivos, e 141 mulheres que tiveram um teste de HPV positivo (com negativo para lesão intraepitelial ou malignidade [NILM] ou inflamação, ou citologia ASC-US e LSIL, mas sem lesões na colposcopia ou ausência de displasia na histologia) repetiram o teste de HPV pelo menos uma vez. Destas 141 mulheres, 6 foram diagnosticadas com lesão intraepitelial escamosa de alto grau (HSIL) durante o período de acompanhamento. Todos os casos de HSIL foram diagnosticados após o segundo teste de HPV.

Conclusão: Este estudo mostra que, no RCCU, todas as mulheres com resultado positivo para HPV, independentemente do resultado do exame de Papanicolaou, devem ser encaminhadas para colposcopia.

Palavras-chave: Cancro do colo do útero; Teste de HPV; Rastreamento de câncer cervical; HSIL; CIN

4.1.2 - Introduction

According to the World Health Organization (WHO) statistics, around 15 to 20% of the diagnosed cancers are associated with viral infections. Human papillomavirus (HPV) is one of the viruses contributing to these statistics, increasing the risk of cervical cancer (CC) progression when high-risk HPV infection persists [125]. CC is the fourth most common cancer in women worldwide, after breast cancer, colorectal cancer and lung cancer [6].

Screening programs which incorporate HPV testing have consistently been associated with a reduction in CC incidence, potentially decreasing morbidity and mortality [125]. Nevertheless, CC remains a major public health problem, with estimated 569,847 new cases and 311,365 deaths worldwide in 2018 [1].

Persistent infection with high-risk HPV genotypes is a necessary but not sufficient condition for disease progression and is the main epidemiological driver of high-grade intraepithelial lesions (HSIL) and invasive carcinoma [130]. HPV infection is subclinical in most cases, especially in younger women where in more than 80% of cases the infection resolves spontaneously within 1 to 2 years. However, approximately 10% of HPV infections can become persistent and about 3 to 4% progress to intraepithelial lesions. Of these, 0.7 to 1% may advance to high-grade lesions (CIN 2/3), being estimated that 0.1% will progress to invasive cancer if not detected and treated in a timely manner [135].

The natural course of CC is well known, and its carcinogenesis process is slow. The presence of CC precursor lesions, the availability of sensitive screening tests for detection and effective treatment methods have enabled highly effective secondary prevention, using screening programs [134].

The most common CC screening methods are conventional cytology, liquid-based cytology and HPV testing, or an association of the latter two [135]. The “standard” screening method has been morphological cytology. Several studies have shown that HPV testing is more sensitive than cytology alone in detecting and preventing

high-grade lesions and progression to cancer. In addition, when using HPV testing as a screening method, the presence of a negative test allows the screening interval to be extended to 5 years, improving compliance with screening programs and enabling effective cost reductions of approximately 20% [121;126;133; 134].

In 2017, a national organized CC HPV-based screening program was implemented in Portugal for women between the ages of 25 and 60 years, performed every 5 years, with reflex cytology for high-risk HPV genotypes other than HPV 16 and 18. This screening program introduces updates to the previous regional cervical cancer screening programs and states that women with a positive HPV test for genotypes other than 16 and 18 with negative for intraepithelial lesion or malignancy (NILM) cytology should repeat the HPV test within the following year. In case of a second HPV test is positive, the woman will be referred for colposcopy. Following 2013 Kaiser Permanent Northern California (KPNC) study results, women with a repetitive positive HPV test with without or minor cytological abnormality (ASC-US/LSIL) and no dysplasia on cervical oriented-colposcopy biopsy should be recommended to colposcopy based on co-testing and Hybrid Capture 2 (HC2; Qliagen, Germantown, MD) for HPV testing [128]. However, no scientific report showed whether this approach is useful on cervical cancer screenings based on primary new molecular technologies for HPV testing with reflex cytology.

Our goal was to use the opportunistic CC screening program of the Cova da Beira University Hospital Center (CHUCB), based on primary COBAS HPV testing and triage cytology, to validate colposcopy recommendation for those women with repeated positive HPV test for genotypes other than HPV 16 and 18 and NILM or minor lesions on previous cytology and no previous dysplasia detected on cervical oriented-colposcopy biopsy.

4.1.3 - Materials and methods

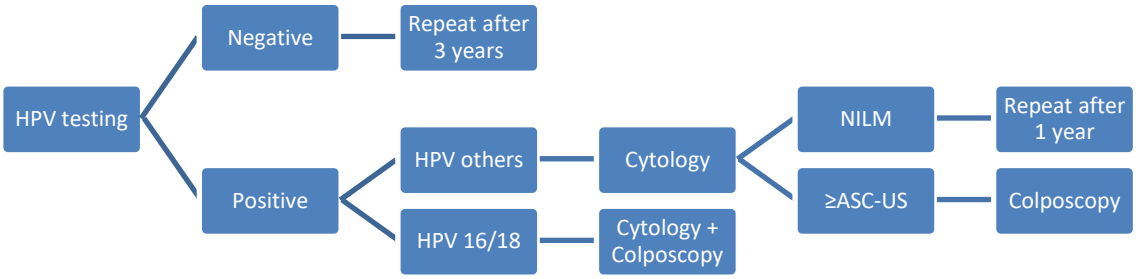
A cross-sectional and retrospective study was carried out based on data from the routine CC screening protocol in force at CHUCB between August 2012 and August 2017. The screening protocol was based on HPV testing as the primary method for all women over 25 years old with no history of CC screening in the past 2 years who attend gynecology appointments at the CHUCB.

The screening method was the Cobas®4800 HPV test, which detects HPV 16, HPV 18 and other types of HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68), using the liquid medium Surepath®.

All HPV tests were performed at the Laboratory of the Clinical Pathology Department of the CHUCB, while the cytological and histological tests were conducted at the Anatomical Pathology Department of the CHUCB.

The CC screening program of the CHUCB was designed and implemented by the CHUCB Colposcopy Unit, where all colposcopic examination were performed. According to the CHUCB screening algorithm, shown in Figure 8, a negative HPV tests should be repeated after 3 years; a positive HPV test for genotypes 16 or 18 is followed by reflex cytology and referral for a colposcopy; a positive HPV test for other types of HPV is followed by reflex cytology, and if reflex cytology shows NILM, the test must be repeated after 1 year; any other cytological finding requires referral for a colposcopy. After a second consecutive positive HPV test, the woman is monitored at the Colposcopy Unit for at least 3 years, regardless of subsequent HPV test results and cytology findings.

Figure/Figura 8 - Flowchart of cervical cancer screening implemented on Cova da Beira University Hospital Center (CHUCB)



Of all 6003 HPV tests performed in 5227 women who underwent routine screening at the CHUCB over the abovementioned 5 years, 765 (14.6%) women who had a positive HPV test were selected for our study. Of these, we evaluated 141 women who had no history of treatment for cervical intraepithelial neoplasia and had satisfactory cytology findings, classified as NILM or minor cytological lesions (ASC-US/LSIL), normal colposcopy and/or no dysplasia on biopsy, and who had follow-up appointments at the Colposcopy Unit of the CHUCB. A biopsy was required during a colposcopy appointment only in the presence of grade 1 or 2 colposcopic findings or signs of invasion. If the transformation zone is classified as type 3 (squamouscolumnar junction not fully visible), endocervical curettage is performed routinely.

A descriptive statistical analysis of the data was performed, using the IBM SPSS application software, version 26 (SPSS Inc., Chicago, IL). In all cases, we analyzed the patient's age, HPV test results (type 16, 18 and others), and cytology and histology findings of the biopsy obtained using colposcopy.

4.1.4 - Results

The study sample consisted of 141 women who had a positive HPV test (to HPV 16, 18 and others) with reflex cytology classified as NILM or minor cytological lesions (ASC-US/LSIL), but normal colposcopy and/or no dysplasia on biopsy

and who underwent follow-up (Table 12). This corresponds to 18.4% of all women with a positive HPV test during the study, aged between 17 and 69, with a mean age of 39.3 years (standard deviation=11.1). For these women, the mean follow-up was 36.6 months (standard deviation=18.5).

	1st Test (N=141)	2nd Test (N=141)	3rd Test (N=55)	4th Test (N=19)	5th Test (N=6)
HPV TEST					
Negative	-	61 (43.3)	20 (36.4)	4 (21.1)	3 (50)
HPV 16	16 (11.3%)	6 (4.2)	2 (3.6)	1 (5.3)	1 (16.6)
HPV 18	5 (3.5)	-	1 (1.8)	-	-
Others	102 (72)	59 (41.8)	26 (47.3)	11 (57.9)	2 (33.4)
HPV 16+others	15 (10.6)	11 (7.8)	5 (9.1)	2 (10.6)	-
HPV 18+others	3 (2.1)	3 (2.1)	1 (1.8)	1 (5.3)	-
CYTOLOGY					
Not performed	1 (0.7)	55(39)	17(31)	1(5.3)	3(50)
NILM	101 (71.7)	51 (36.2)	28 (50.9)	13 (68.4)	2 (33.3)
LSIL	24 (17.0)	13 (9.3)	3 (5.4)	2 (10.5)	-
ASC-US	15 (10.6)	15 (10.6)	6 (10.9)	2 (10.5)	1 (16.7)
HSIL	-	3 (2.1)	1 (1.8)	1 (5.3)	-
ASC-H	-	4 (2.8)	-	-	-
HISTOLOGY					
Not performed	24 (53.3)	104 (73.7)	49 (89.1)	14 (73.7)	4 (66.6)
No dysplasia	21 (46.7)	18 (12.8)	4 (7.3)	3 (15.8)	1 (16.7)
LSIL	-	13 (9.2)	2 (3.6)	2 (10.5)	1 (16.7)
HSIL	-	6 (4.3)	-	-	-

Table/Tabela 12 - Sequency of human papillomavirus (HPV) tests, cytology and histology results. Data is presented as number (percentage, %)

During follow-up, CIN2+ lesions were detected in six (4.3%) women, with a mean age of 35.7 years (standard deviation= 7.7), and all CIN2+ lesions were diagnosed after the second HPV test. No women were diagnosed with invasive carcinoma. The mean time to diagnosis of CIN2+ lesions was 18.5 months (standard deviation=4.2). The HPV test, cervical cytology and biopsy results are shown in Table 12. Regression rate of HPV infection in the studied group was always very high, especially for types 16 and 18, which highlights the transient nature of those HPV infections. However, the multiple infection rate (HPV 16 or 18 and others) remained unchanged, possibly due to reinfection. Following the first test, only 45 women underwent colposcopy due to a positive HPV 16 or 18 test and/or ASC-US or LSIL cytology. All women underwent colposcopy in their second, third, fourth

and fifth HPV tests. Six women were co-tested for their second HPV test, and 3 women were co-tested for their third and fourth tests.

Table 13 shows that the prevalence and spontaneous resolution of high-risk HPV infection was more common in women under 30 years of age, while the cytological and histological diagnosis was more serious in the group of women over 30 years of age.

	1st Test		2nd Test		3rd Test		4th Test		5th Test	
Age	<30	>30	<30	>30	<30	>30	<30	>30	<30	>30
Number of cases	30 (21.3)	111 (78.7)	30 (21.3)	111 (78.7)	11 (20)	44 (80)	2 (1.1)	17 (98.9)	-	6 (100)
HPV TEST										
Negative	-	-	15 (50)	46 (41.4)	4 (36.4)	16 (36.3)	1 (50)	3 (17.6)	-	3 (50)
HPV 16	8 (26.7)	8 (7.2)	1 (3.3)	5 (4.5)	1 (9.1)	1 (2.3)	-	1 (5.9)	-	1 (16.7)
HPV 18	-	5 (4.5)	-	-	1 (9.1)	-	-	-	-	-
Others	19 (63.3)	83 (74.8)	9 (30)	50 (45)	5 (45.4)	21 (47.7)	1 (50)	10 (58.8)	-	2 (33.3)
HPV 16+HPV 18	-	-	-	1 (0.9)	-	-	-	-	-	-
HPV 16+others	3 (10)	12 (10.1)	4 (13.3)	7 (6.3)	-	5 (11.4)	-	2 (11.8)	-	-
HPV 18+others	-	3 (2.7)	1 (3.3)	2 (1.8)	-	1 (2.3)	-	1 (5.9)	-	-
CYTOLOGY										
Not performed	1 (3.3)	-	14 (46.7)	41 (37)	3 (27.3)	14 (31.8)	1 (50)	-	-	3 (50)
NILM	20 (66.7)	81 (73)	12 (40)	39 (35)	8 (72.7)	20 (45.5)	1 (50)	12 (70.6)	-	2 (33.3)
LSIL	6 (20)	18 (16.2)	2 (6.7)	11 (10)	-	3 (6.8)	-	2 (11.8)	-	-
ASC-US	3 (10)	12 (10.8)	1 (3.3)	14 (12.6)	-	6 (13.6)	-	2 (11.8)	-	1 (16.7)
HSIL	-	-	1 (3.3)	2 (1.8)	-	1 (2.3)	-	1 (5.8)	-	-

ASC-H	-	-	-	4 (3.6)	-	-	-	-	-	-
HISTOLOGY										
Not performed	6 (20)	18 (16.2)	24 (80)	80 (72)	9 (81.8)	40 (91)	1 (50)	12 (70.6)	-	4 (66.6)
No dysplasia	3 (10)	18 (16.2)	2 (6.7)	14 (12.6)	2 (18.2)	2 (4.5)	1 (50)	3 (17.6)	-	1 (16.7)
LSIL	-	-	-	13 (11.7)	-	2 (4.5)	-	2 (11.8)	-	1 (16.7)
HSIL	-	-	2 (6.7)	4 (3.6)	-	-	-	-	-	-

Table/Tabela 13 - Sequency of human papillomavirus (HPV) tests, cytology and histology results comparing women under and over 30 years of age. Data is presented as number (percentage, %)

Table 14 shows the relevant aspects of the 6 cases where HSIL was diagnosed during patient follow-up. Four of these 6 cases were diagnosed in women aged 30 or over. HSIL was associated with HPV 16 infection in only one woman, and cytology had been classified as NILM or ASC-US or LSIL in 4 women.

Description of positive cases						
Age	1st HPV Test	1st Cytology	2nd HPV Test	2nd Cytology	Time to diagnosis	Notes
25 years old	16 + Others	NILM	Others	HSIL	18 months	
27 years old	16 + Others	LSIL	16 + Others	LSIL	22 months	1)
30 years old	Others	NILM	Others	HSIL	25 months	
40 years old	Others	NILM	Others	NILM	16 months	2)
42 years old	Others	LSIL	Others	ASC-US	14 months	
50 years old	Others	LSIL	Negative	ASC-US	16 months	3)

Table/Tabela 14 - Detailed description of high-grade squamous intraepithelial

4.1.5 - Discussion

The protocol used in this study was the CC screening protocol of the Gynecology Department of the CHUCB, which recommends HPV testing as the primary test in routine screening. This is an institutional screening program which, among other aspects, is open to all patients attending gynecology appointments (including pregnant women) and had the participation of all physicians who offer gynecology appointments at the CHUCB. This cervical cancer screening was

implemented at the CHUCB to manage patients and to mitigate the effects of low compliance with the national screening program.

The CC screening protocol at the Gynecology Department of the CHUCB beginning at 2012 was organized following 2011 ATHENA HPV study results [39].

A high percentage of women under the age of 30 were included in the study population. The CC screening protocol in force at the CHUCB includes women over 25 years of age and some physicians did not comply with the inclusion criteria. There was a higher prevalence and spontaneous resolution of high-risk HPV infection in the group of women under 30 years of age, as well as less serious cytological and histological diagnoses, which is in accordance with the literature.

The HPV test used for screening was the Cobas®4800 HPV test, which is a qualitative test that uses real-time PCR technology to simultaneously detect DNA from 12 types of human recombinant HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and individually detect HPV 16 and 18. β-globin gene is amplified as an internal control. It can be used as a primary screening method with reflex cytology for positive HPV or in addition to cytology (co-testing). Thus, CIN3+ risk stratification is improved, increasing sensitivity for early detection of cervical cancer, with a negative predictive value very close to 100% [127].

The liquid medium used for transport and preservation of all samples for cytology was SurePath®, which does not exhibit significant differences in terms of cut-off values, when compared to other certified liquid collection media, for the detection of CIN1, CIN2+ and CC lesions [132].

Some women who had a positive HPV test did not undergo follow-up because they had a surgery for a benign condition (uterine fibroids or pelvic organ prolapse corrections), or they stopped attending appointments.

In accordance with literature, the percentage of multiple infections was different according to age, suggesting transient reinfection rather than a persistent infection [131].

From the analysis of the 6 cases of HSIL diagnosed, we highlight the importance of performing colposcopy after the second test, as all our cases were diagnosed at this time. Our results are validated by other studies reporting similar situations [127; 129]. The second cytology was suggestive of HSIL in only two cases, and it was classified as NILM in one case, which reinforces the value of colposcopy in these situations.

The absence of HSIL diagnosed after the second HPV test is probably due to the referral for colposcopy of all patients after the second positive HPV test, regardless of cytology findings. This procedure allowed HSIL identification which was not diagnosed during the first test. Between the first and second tests, it is more likely that there was regression than progression of dysplastic lesions, which may also explain in part why no other cases of HSIL were diagnosed after the second test. The outcome of any HPV-based CC screening is highly dependent on the number of lesions detected using colposcopy-directed biopsy, which reinforces the importance of quality colposcopy practices. Avoiding unnecessary biopsies without neglecting the diagnosis of cervical cancer precursor lesions is of paramount importance, and all women with positive HPV tests should be referred to different colposcopy units, as was the case in this study.

This study demonstrates that women undergoing HPV-based CC screening who had one positive HPV test with NILM, ASC-US or LSIL cytology, with normal colposcopic findings and/or no dysplasia on cervical biopsy, should be referred for colposcopy in the presence of a second positive HPV test, regardless of the cytology findings. This procedure is standardized in the current cervical cancer screening program in Portugal and recommended by 2019 American Society for Colposcopy and Cervical Pathology (ASCCP) guidelines [133].

This study has some limitations. The CC screening method used at the CHUCB is an institutional routine program based on a random population that attends gynecology appointments and includes pregnant women, and it is not an organized screening program. Furthermore, sample size is limited as the geographic localization of CHUCB only serves a population of approximately 90,000 people which includes the municipalities of Covilhã, Fundão, Belmonte

and Penamacor. Only women referred by CHUCB physicians to the CHUCB Colposcopy Unit were evaluated in this study. Many of these women had previously participated in the organized cervical cancer-screening program in the Centre Region of Portugal, which has been in place for more than 20 years. The influence of HPV vaccination on the results was not evaluated because the percentage of vaccinated women was small at the time of data collection.

Nevertheless, the results of our study concerning HPV and cytology abnormalities prevalence are in agreement with studies performed in other countries, such as the HEllenic Real life Multicentric cErvical Screening study group, in Greece (HERMES) [121] and a study that evaluates the efficacy outcomes of primary HPV testing based on the follow-up of randomized controlled trials in Germany (WOLPHSCREEN), Sweden (SWEDESCREEN), England (ARTISTIC), the Netherlands (POBASCAM) and Italy (NTCC) [89]. Therefore, we can conclude that our studied population was adequate for valid conclusions. In addition, our study is in agreement with recent published 2019 ASCCP Risk-Based Management Consensus Guidelines for Abnormal Cervical Cancer Screening Tests and Cancer Precursors [122], that recommend colposcopies for all women with repeated positive HPV testing with NILM or minor cytology abnormalities.

This study has shown that, regardless of reflex cytology findings, women who have at least two consecutive positive cervical HPV tests are at increased risk of having previously undiagnosed cervical HSIL and should always be referred for colposcopy. Additionally, the risk of intraepithelial lesions or malignancy was independent of the type of HPV determined. All women with cervical repeated positive HPV testing and with absent or minor cytology abnormalities should be referred to colposcopy in an independent way of screening adopted program and technology used for HPV testing, as recommended by ASCCP.

List of abbreviations: ASC-H—atypical squamous cells; CC—cervical cancer; CHUCB—Cova da Beira University Hospital Center; CIN—cervical intraepithelial neoplasia; HPV—human papillomavirus; HSIL—high-grade squamous intraepithelial lesion; NILM—negative for intraepithelial lesion or malignancy; SPSS—Statistical Package for the Social Sciences.

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Conflict of interest

The authors have no conflict of interest to declare. This research was approved by the Ethics Committee of Beira Interior University with the code CE-UBI-Pj-2017-027.

Author contribution

Vitor Caeiro: Conducted the investigation and write manuscript; Sara Nunes: Organize statistical analysis of data and review the manuscript; Bruno Esteves: Responsible by HPV testing and pap smear and review the manuscript; José Fonseca-Moutinho; Advisor and review the manuscript.

4.2 - HPV testing for cervical cancer screening: Should reflex cytology be performed after a positive test for HPV 16 and 18?

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4.2.1 - Abstract

At Portuguese-organized cervical cancer (CC) screening programs, all women testing positive for human papillomavirus (HPV) 16 and/or 18 are referred for immediate colposcopy. This study aimed to evaluate the utility of reflex cytology in women who test positive for HPV 16 and/or 18 to improve the efficiency of CC screening.

A cross-sectional and retrospective study was performed based on data from the routine CC screening protocol in force at Cova da Beira University Hospital Center, Portugal between August 2012 and June 2021. The screening method was the Cobas 4800 HPV test using the liquid medium Surepath. In all the selected cases, the patient's HPV test results and the cytology and histology findings of the biopsies obtained using colposcopy were analyzed.

This study included 339 women who first tested positive for HPV 16 and/or 18 and were referred for immediate colposcopy, in whom 40 (11.8%) cases of high-grade squamous intraepithelial lesion (HSIL+) were diagnosed. Of these, 12 (30%) had reflex cytology negative for intraepithelial lesion or malignancy (NILM) and 14 (35%) had HSIL+ cytology. After 3 years, 14 (9.3%) of the 150 women who were still undergoing follow-up were diagnosed with histologic HSIL+ lesions, of which 5 (35.7%) had baseline NILM cytology.

Despite the small sample, the results of this study allow us to conclude that reflex cytology is not useful for discrimination to immediate referral for colposcopy in women who test positive for HPV 16 and/or 18, as most women with a histologic diagnosis of an HSIL+ lesion had <HSIL reflex cytology.

Key Words: reflex cytology, HPV, colposcopy, HSIL, cervical cancer

Resumo

Nos programas de rastreio do cancro do colo do útero (CCU) organizados em Portugal, todas as mulheres com resultados positivos para o papilomavírus humano (HPV) 16 e/ou 18 são encaminhadas para colposcopia imediata. Este estudo teve como objetivo avaliar a utilidade da citologia reflexa em mulheres com teste positivo para HPV 16 e/ou 18 para melhorar a eficiência do rastreio do CCU.

Foi realizado um estudo transversal e retrospetivo com base nos dados do protocolo de rastreio de rotina do CCU em vigor no Centro Hospitalar Universitário Cova da Beira, Portugal, entre agosto de 2012 e junho de 2021. O método de rastreio foi o teste Cobas 4800 HPV utilizando o meio líquido Surepath. . Em todos os casos selecionados, foram analisados os resultados do teste de HPV do paciente e os achados citológicos e histológicos das biópsias obtidas por colposcopia.

Este estudo incluiu 339 mulheres que inicialmente testaram positivo para HPV 16 e/ou 18 e foram encaminhadas para colposcopia imediata, nas quais foram diagnosticados 40 (11,8%) casos de lesão intraepitelial escamosa de alto grau (HSIL+). Destes, 12 (30%) tiveram citologia reflexa negativa para lesão intraepitelial ou malignidade (NILM) e 14 (35%) tiveram citologia HSIL+. Após 3 anos, 14 (9,3%) das 150 mulheres que ainda estavam em acompanhamento foram diagnosticadas com lesões histológicas HSIL+, das quais 5 (35,7%) tinham citologia NILM basal.

Apesar da pequena amostra, os resultados deste estudo permitem concluir que a citologia reflexa não é útil para discriminar o encaminhamento imediato para colposcopia em mulheres com teste positivo para HPV 16 e/ou 18, pois a maioria das mulheres com diagnóstico histológico de HSIL+ apresentava citologia reflexa <HSIL.

Palavras-chave: Citologia reflexa; HPV; Colposcopia; HSIL; cancro do colo do útero

4.2.2 - Introduction

Cervical cancer (CC) is the fourth most common cancer in women worldwide, following breast cancer, colorectal cancer. and lung cancer [1,6] Persistent infection with high-risk HPV genotypes is the main epidemiological factor responsible for high-grade intraepithelial lesions (HSIL) and invasive carcinoma; however, it is not a determinant condition [8].

Early CC screening is very helpful in the management of disease severity, with conventional cytology, liquid-based cytology, and HPV testing, or a combination of the latter two, being the most used methods [136].

Detection and prevention of high-grade lesions are shown to be more effective with HPV testing rather than cytology alone. Moreover, HPV testing that has a negative result allows longer screening intervals (up to 5 years), improving compliance and reducing the associated cost by approximately 20% [121; 126; 133; 134]

In 2017, a nationally organized CC HPV-based screening program was implemented in Portugal for women between the ages of 25 and 60 years, performed every 5 years, with reflex cytology for high-risk HPV genotypes other than HPV 16 and 18. This screening program introduces updates to the previous regional cervical cancer screening programs and states that women with a positive HPV test for genotypes other than 16 and 18 with cytology negative for intraepithelial lesion or malignancy (NILM) should repeat the HPV test within the following year. Immediate referral for colposcopy in women who test positive for HPV 16 and 18 was based on the findings of increased risk for HSIL+ lesions, evidenced in the ATHENA [39], ARTISTIC [105], and KPNC [137] studies. However, most women who test positive for HPV genotypes 16 and 18 and are immediately referred for colposcopy are not diagnosed with an HSIL+ lesion, meaning that they unnecessarily undergo a procedure with some morbidity.

In the KPNC and Athena studies, in cases where cotesting revealed a positive HPV test for genotypes 16 and 18 and HSIL cytology, the risk of immediate HSIL lesions was 40%, compared with approximately 10% in women with <HSIL cytology [39, 137] which suggests that reflex cytology, in these cases, could inform colposcopy practice. In Portugal, as the national cervical cancer screening program does not provide for cytology (cotesting or reflex testing), the potential interest of performing reflex cytology in cases of positive HPV 16 and 18 testing has not yet been determined.

This study aims to evaluate the usefulness of reflex cytology in women who test positive for HPV 16 and/or 18, in order to improve the efficiency of Portuguese CC screening.

4.2.3 - Materials and Methods

A cross-sectional and retrospective study was carried out based on data from the routine CC screening protocol in force at Cova Beira Hospital Center (CHUCB) between August 2012 and June 2021. The study was reviewed and approved by the Ethics Committee of the University of Beira Interior and carried out in accordance with relevant guidelines and regulations.

CHUCB's catchment area includes the municipalities of Covilhã, Fundão, Belmonte, and Penamacor, and the hospital center provides care to a population of approximately 90,000, so the sample size was limited.

4.2.3.2 - Participants

Data on all women who attended gynecology appointments at the CHUCB who were over 25 years old and had no history of CC screening in the past 2 years were included in this study.

Of all 8022 HPV tests performed on 6376 women who underwent routine screening at the CHUCB over the above-mentioned 9 years (August 2012 to June 2021), all 339 (5.3%) women who first tested positive for HPV genotypes 16 and/or 18 and underwent reflex cytology were selected for our study.

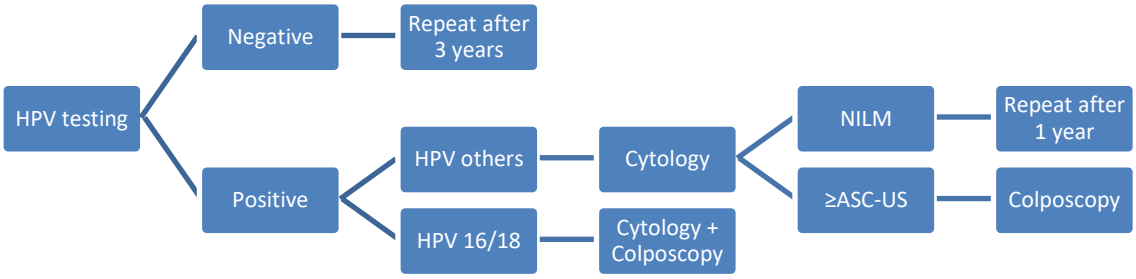
In all these cases, we analyzed the patient's HPV test results (type 16 and/or 18) and cytology and histology findings of the biopsies obtained using colposcopy (immediately and after 3 years).

For the data analysis, as there were only two cases of atypical squamous cells (ASC-H), these were included in the HSIL+ cytology.

4.2.3.3 - Variables and data source

The screening protocol used in this study followed the routine screening algorithm implemented at CHUCB in 2012 (Figure 9). Thus, all women with positive HPV-HR tests underwent reflex cytology, and those who tested positive for HPV genotypes 16 and 18 were also referred for colposcopy.

Figure/Figura 9 - Flowchart of cervical cancer screening implemented on Cova da Beira University Hospital Center (CHUCB)



HPV testing was used as the primary method and performed with a Cobas 4800 HPV test, using the liquid medium Surepath. By using real-time PCR technology and the β-globin gene as an internal control for the test, this qualitative analysis is used to simultaneously detect DNA from 12 types of human recombinant HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and individually detect HPV 16 and 18. HPV testing can be used as a primary screening method with reflex cytology for positive HPV or in addition to cytology (cotesting). Previous studies showed that HPV testing improves CIN3+ risk stratification, which allows early detection of cervical cancer, with increased sensitivity and negative predictive values very close to 100% [90].

All HPV tests were performed at the laboratory of the Clinical Pathology Department of the CHUCB, while cytologic and histologic testing were conducted at the Anatomical Pathology Department of the CHUCB.

The CC screening program was designed and implemented by the CHUCB Colposcopy Unit, where all colposcopies were performed.

According to the CHUCB screening algorithm, shown in Figure 9, a positive HPV test for genotypes 16 or 18 is followed by reflex cytology and referral for a colposcopy. Only patients with abnormal colposcopic findings underwent biopsy, while the remaining patients were instructed to undergo a repeat HPV test within one year.

4.2.3.4 - Statistical analysis

Data were entered and analyzed using SPSS Statistics for Windows, Version 21.0 (Armonk, NY: IBM Corporation). Proportions, arithmetic means, medians, and standard deviations (SD) were used as summary statistics.

4.2.4 - Results

The study sample consisted of 339 women who first tested positive for HPV genotypes 16 and/or 18 with reflex cytology (Table 15).

		Baseline reflex cytology			
HPV test		NILM	LSIL	ASC-US	HSIL+
HPV 16	163	106	25	16	16
HPV 18	28	18	4	2	4
HPV 16+HPVP	107	56	27	16	8
HPV 18+HPVP	32	23	6	3	-
HPV 16+18	5	3	2	-	-
HPV 16+18+HPVP	4	2	-	1	1
TOTAL	**339**	**208**	**64**	**38**	**29**

Table/Tabela 15 - HPV test and cytology results (n = 339)

In these women, all of whom underwent immediate colposcopy, 40 (11.8%) cases of HSIL+ lesion were diagnosed, of which 3 were invasive carcinomas (Table 16).

		Histology (immediate)			
HPV test		Not performed	No dysplasia	LSIL	HSIL+
HPV 16	163	117	10	21	15
HPV 18	27	19	1	4	3
HPV 16+HPVP	107	78	2	11	16
HPV 18+HPVP	33	22	1	6	4
HPV 16+18	5	4	-	1	-
HPV 16+18+HPVP	4	2	-	-	2
Total	339	242	14	43	40

Table/Tabela 16 - Baseline HPV test and cytology results (n = 339)

The mean age of the 6376 women included in the screening program was 43 years (standard deviation 13.03), and the mean age of the 339 women who tested positive for HPV genotypes 16 and/or 18 and were included in the study was 39.7 years (standard deviation 11.51).

Of the 279 women who tested positive for HPV genotype 16, 25 (9%) had HSIL+ cytology, and 254 (91%) had ≤HSIL+ cytology. Seventy-six of these 279 patients underwent colposcopy-directed biopsy, of whom 33 (11.8%) had histologic HSIL+ and the remaining 43 had histologic ≤HSIL+ (Tabela 16 and 17).

		Histology findings at 3 years			
Baseline reflex cytology		Not performed	No dysplasia	LSIL	HSIL+
NILM	104	88	2	9	5
ASC-US	25	22	1	1	1
LSIL	17	11	-	1	5
HSIL+	4	1	-	-	3
Total	150	122	3	11	14

Table/Tabela 17 - Histology findings during 3 years of follow-up

Of the 60 women who tested positive for HPV genotype 18, 4 (7%) had HSIL+ cytology and 56 (93%) had ≤HSIL+ cytology. Nineteen (31.6%) of these 60 women underwent colposcopy-directed biopsy; 7 (11.7%) had histologic HSIL+ and the remaining 12 had histologic ≤HSIL+ (Tables 16 and 18).

		Baseline histology			
Baseline reflex cytology		Not performed	No dysplasia	LSIL	HSIL+
NILM	208	167	10	19	12
ASC-US	38	25	1	4	8
LSIL	64	37	3	18	6
HSIL+	29	13	-	2	14
Total	339	242	14	43	40

Table/Tabela 18 - Baseline cytology and histology results.

After 3 years, 92 of the 242 women who did not undergo a biopsy at baseline were lost to follow-up due to hysterectomy for a benign condition, pregnancy, or drop-out. Fourteen (9.3%) of the 150 women who continued to undergo follow-up were diagnosed with histologic HSIL+ lesions during the following 3 years of follow-up. Of these 14 patients, 5 (35.7%) had baseline NILM cytology (Table 17).

Table 18 shows the histologic findings of the colposcopy-directed biopsies. We can see that 12 (30%) of the 40 patients diagnosed with HSIL+ had NILM reflex cytology and only 14 (35%) had HSIL+ cytology.

	BASELINE HISTOLOGY		
BASELINE REFLEX CYTOLOGY		**Positive (LSIL+HSIL)**	**Negative**
	Positive	**40**	**53**
	Negative	**43**	**203**

Table/Tabela 19 - Specificity—sensitivity of reflex cytology and histology
Sensibility: 48%; Specificity: 79%; Accuracy: 71%

	Baseline histology		
Colposcopic findings	**No displasia**	**LSIL**	**HSIL+**
Normal	-	-	-
Grade 1	10	35	7
Grade 2	0	8	33
Total	10	43	40

Table/Tabela 20 - Colposcopic findings and histology results

4.2.5 - Discussion

This study, based on data from an opportunistic cervical cancer screening institutional program (CHUCB) that took place between August 2012 and March 2021, showed that reflex cytology, in the presence of a positive HPV test for types 16 and 18, does not inform colposcopy practice, as most HSIL+ lesions diagnosed immediately or after 3 years of follow-up were diagnosed in women with <HSIL reflex cytology.

Surepath, the liquid medium used for transport and preservation of all samples for cytology, does not seem to introduce significant differences in terms of cutoff values when compared with other certified liquid collection media for the detection of CIN1, CIN2+, and CC lesions [132].

Three hundred thirty-nine (5.3%) of the 6376 women included in the CHUCB screening program (from August 2012 to March 2021) tested positive for HPV genotypes 16 and/or 18, which does not differ significantly from the number seen in other studies, such as ATHENA [39] and KPNC, [137] although there were significant differences in the characteristics of the populations involved. Of these women, 40 (11.8%) had baseline histologic HSIL+. Only 14 (4.1%) had histologic HSIL+ over 3 years, and these results are in agreement with the studies reported in the literature. Of the 29 women with baseline HSIL+ cytology, only 14 (48.3%) had baseline histologic HSIL+.

Only women with abnormal colposcopic findings underwent biopsy, in contrast with other studies, in which a biopsy was performed in all cases. However, "blind" biopsies (4-quadrant biopsies) have not been shown to be more advantageous than colposcopy-directed biopsies, as reported by some studies [138,139].

All colposcopic examinations were performed by a single observer with accreditation by the Portuguese Colposcopy Society, so there is always the possibility of individual bias, which may explain the low number of biopsies performed. However, there were a very small number of biopsies with no dysplasia, which suggests that there is some selectivity as to whether a biopsy is performed. It is possible that some of the HSIL lesions diagnosed in the 3 years following the first colposcopy were already present at the time and were not diagnosed, but it should be noted that in this situation, approximately 97.3% of the women had a baseline <HSIL reflex cytology.

In most of the studies published on this subject, it seems evident that there is an increased risk of histologic HSIL+ lesions for HPV-16 positive cases compared with HPV 18; in our study, this difference does not appear significant, possibly due to the small size of the sample and the characteristics of its population.

The risk of having an HSIL lesion after a positive HPV-16 and/or -18 test is greater than 4%; therefore, according to the 2019 ASCCP Guidelines [122], immediate colposcopy referral is always recommended, with no need for reflex cytology, which was not useful in informing colposcopy practice.

4.2.6 - Conclusions

Despite the small sample and some limitations of the study, the results are nevertheless sufficient to draw some conclusions.

The assessment of the risk of HSIL lesions in the CC screening program has been fundamentally based on programs that use cotesting, with little evidence of this risk in screening programs that rely on the HPV test.

In a nationwide CC screening program, immediate colposcopy referral for all women who test positive for HPV 16 and 18 requires considerable effort. It is tempting to stratify these women into low- and high-risk groups through reflex cytology. However, this study found that reflex cytology seems not to be useful for immediate referral for colposcopy in women who test positive for HPV 16 and/or 18, as most women with histologic diagnosis of an HSIL+ lesion had <HSIL reflex cytology.

List of abbreviations: ASC-H—atypical squamous cells; CC—cervical cancer; CHUCB—Cova da Beira University Hospital Center; CIN—cervical intraepithelial neoplasia; HPV—human papillomavirus; HSIL—high-grade squamous intraepithelial lesion; NILM—negative for intraepithelial lesion or malignancy; SPSS—Statistical Package for the Social Sciences.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgments

The authors would like to thank CHUCB for providing the facilities for this study.

Ethics Approval

The Ethics Committee of the University of Beira Interior approved the request for an opinion on this study, to which it assigned code CE-UBI-Pj-2017-027.

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Highlights

- Immediate colposcopy referral is a considerable effort for health care systems.
- Stratifying women into low- and high-risk groups through reflex cytology is tempting.
- In this study, reflex cytology was not useful for immediate referral for colposcopy.
- Most women with histologic diagnosis of an HSIL+ lesion had <HSIL reflex cytology.

4.3 - High-Risk HPV Detection in Paraffin-Embedded Tissue from Cervical Lesions.

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4.3.1 - Abstract

Background: Human papillomavirus (HPV) is diagnosed in most cervical cancers and is the fourth cause of cancer worldwide. Currently, fourteen HPV types are considered carcinogenic (HPV 16/18/31/33/35/39/45/51/52/56/58/59/66/68) with different oncogenicity. Cervical lesions might be due to HR (high risk)-HPV for what is important to identify new methods to genotype cervical lesions.

Methods: The main goal was to identify, for the first time, HPV-HR in cervical lesions using Anyplex™ Detection kit in paraffin-embedded tissue. It was performed a retrospective study in 45 women from Cova da Beira Local Health Unit. DNA extraction was performed and through Anyplex™ II HPV-HR Detection kit

Results: HPV-HR were genotyped. HPV-HR were identified in 38 women: HPV-16 (55,26%), HPV-18/39/56/58/59 (5,26%), HPV-31 (21,05%), HPV-35 (7,89%), HPV-51/66 (2,63%) and HPV-52 (10,53%).

Conclusions: Our results reflect that Anyplex™ II HPV-HR Detection kit, designed for HR-HPV genotyping of cervical cancer screening, might be properly used to HR-HPV genotyping in histology. The proposed methodology allows an easy and cheaper technique that should be used as a future cervical risk stratification. It is a strong tool to be implemented in clinical practice in order to detect HR-HPV detection in cervical lesions, contributing to a more accurate diagnosis.

Keywords: HR-HPV Detection kit; HR-HPV genotyping; Cervical Cancer

Resumo

Antecedentes: O vírus do papiloma humano (HPV), uma das principais causas de cancro do colo do útero (CCU), está presente na maioria dos casos da doença e é o quarto cancro mais comum nas mulheres no mundo. Entre os tipos de HPV, catorze (HPV 16/18/31/33/35/39/45/51/52/56/58/59/66/68) são reconhecidos como de alto risco (HPV-hr), cada um com diferentes níveis de potencial oncogénico. A deteção e determinação do genótipo destes tipos de HPV-hr em lesões cervicais é crucial, exigindo o desenvolvimento de novos métodos de diagnóstico.

Métodos: Este estudo centra-se numa análise retrospetiva realizada a 44 mulheres da Unidade Local de Saúde da Cova da Beira. Utilizámos o kit de deteção de HPV-hr Anyplex™ II para a determinação do genótipo de HPV-hr a partir de amostras de tecido cervical embebidas em parafina.

Resultados: Os tipos de HPV-hr foram identificados em 38 mulheres. A determinação do genótipo revelou HPV-16 (55,3%), HPV-18/39/56/58/59 (5,3%), HPV-31 (21,1%), HPV-35 (7,9%), HPV-51/66 (2,6%) e HPV-52 (10,5%).

Conclusões: Este estudo demonstra que o kit de deteção de HPV-hr Anyplex™ II, originalmente concebido para o rastreio do CCU, também é eficaz para a determinação do genótipo de HPV-hr em análises histológicas. Esta metodologia oferece uma abordagem mais simples e mais económica para a estratificação do risco de CCU. A sua implementação na prática clínica poderá melhorar a deteção de HPV-hr em lesões cervicais, contribuindo assim para diagnósticos mais precisos e estratégias de tratamento potencialmente mais informadas.

Palavras-chave: cancro do colo do útero, Kit de deteção de HPV-hr; Determinação do genótipo do HPV-hr.

4.3.2 - Introduction

Human papillomavirus (HPV) is diagnosed in more than 90% of cervical cancers [3], being the fourth cause of cancer in women [140]. This disease leads to a substantial mortality rate, being the foremost cause of cancer-related deaths in women across 23 countries, making it a significant concern in women's health globally [140].

The International Agency for Research on Cancer (IARC) categorizes certain types as high-risk (HR-HPV) due to their strong association with cervical cancer [141]. Specifically, HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 are classified under Group 1, indicating a direct carcinogenic risk to humans [141]. Meanwhile, HPV-68 and HPV-66 are deemed as probably carcinogenic (Group 2A) and possibly carcinogenic (Group 2B), respectively, suggesting a varying degree of risk in contributing to cervical cancer development [141].

The implementation of HPV cytological screening programs, including both conventional cytology and liquid-based cytology, has played a pivotal role in reducing the incidence of cervical cancer [4, 5]. These screening methods have significantly improved the early detection of cervical precancerous lesions and significantly contributed to cervical cancer incidence reduction [4, 5]. However, the development of HPV testing for the detection of HR-HPV types offers a more sensitive approach for identifying high-grade squamous intraepithelial lesions (HSIL), surpassing the diagnostic capabilities of cytology. As such, HPV testing for HR-HPV (HR-HPV-16/18/31/33/35/39/45/51/52/56/58/59/66/68) is now recommended as the primary cervical cancer screening test by nearly all scientific societies' guidelines [142]. This shift towards HPV testing reflects an evolving understanding of the critical role that HR-HPV types play in the etiology of cervical cancer and the need for an earlier and accurate detection.

In alignment with the screen-and-treat strategies endorsed by the World-Health Organization (WHO), visual inspection with acetic acid (VIA) and HR-HPV DNA tests are recommended [5]. These HR-HPC DNA tests are designed to identify the 14 types of HR-HPV associated with a higher risk of cervical cancer [5]. Usually, the assessment of cancer risk is based on HPV testing and reflex cytology, if a determined risk is higher than 4 or 5%, the women is oriented to immediate colposcopy and biopsy or excision of transformation zone [143].

Despite these advances, challenges remain, particularly for histologic high-grade cervical lesions, once there are no validated tools to assess the risk of progression for cancer. Lower age (less than 25-30 years old) have been used as a criteria for no treatment [144].

Given the more aggressive behavior of HPV type 16 and HPV type 18 and the link with an increased risk of invasive cancer progression [145], the determination of HPV type in histologic legions of HSIL emerges as a potential tool for risk stratification and treatment optimization.

The development of accessible and cost-effective techniques for HPV genotyping in histologic lesions of HSIL is crucial for the clinical practice.

While HR-HPV tests are commonly used for genotyping samples collected via Pap tests, identifying HR-HPV genotypes in existing lesions remains a significant need. This gap underscores the importance of advancing screening programs and HR-HPV DNA tests to improve the detection of the HR-HPV genotypes and the clinical management when the precancerous lesion have already occurred.

The main goal of this work is to stablish, for the first time, a protocol to HR-HPV detection in cervical lesions assembled in paraffin-embedded tissue samples using the detection kit Anyplex™ II HPV HR Detection kit (Seegene®, Seoul, Korea, acquired to Werfen, Portugal) originally designed for cervical cancer screening. This approach represents a novel application in the context of HR-HPV identification when the lesion has already occurred. The successful implementation of this protocol could significantly enhance diagnostic accuracy, leading to a more effective management strategies of cervical cancer. Moreover, this advancement has the potential to improve prognosis and patient outcome substantially. In the future, the incorporation of this protocol into existing algorithms of HPV screen-and-treat approaches could prompt updates to HPV screening guidelines, marking a significant step forward in the fight against cervical cancer.

4.3.3 - Materials and Methods

4.3.3.1 - Study population

A retrospective study was performed using paraffin-embedded tissue samples from 45 women who had previously undergone to the excision of squamous high-grade cervical lesions at the Cova da Beira Local Health Unit. The samples were collected at the Child and Women, Gynaecologic Oncology Division of Cova da Beira Local Health Unit,

Covilhã, Portugal. The study was approved by the Ethics Committee of Beira Interior University with the code CE-UBI-Pj-2017-027.

4.3.3.2 - DNA Extraction

The meticulous process of DNA extraction from tissue samples embedded in paraffin is a critical step for the molecular analysis.

Initially, prior to DNA extraction, 3µm thick slides were stained with hematoxylin eosin. The slides were assessed by two independent pathologists in order to confirm the presence of HSIL.

Upon confirmation of HSIL, the procedure of DNA extraction was performed.

The process of preparing the slides for DNA extraction involves several meticulous executes step to prevent cross-contamination and ensure the purity of the DNA.

The first step for DNA extraction optimization was the thickness of the tissue, thus 10µm paraffin-embedded biopsy slides were obtained for each confirmed case of HSIL.

Following the xylene treatment, the slides were submerged in xylene until the slide was completely covered. The incubation was carried out using a new sterile falcon tube, of 50mL for each slide in order to avoid cross-contamination. This step is crucial for deparaffinization, ensuring that the paraffin. Which embeds and preserves the tissue is completely dissolved, allowing the exposure of the underlying tissue.

The slides were then individually transferred to a new 50mL sterile falcon tube and absolute ethanol was used until the slide was completely submerged. The incubation period was of 5min at room temperature. After, the slides were allowed to dry at room temperature for 5 to 10min.

The next step involves the physical retrieval of the tissue from the slide. A new sterile scalpel was used for each sample, the tissue was carefully scraped off the into a 1,5mL microtube. This is a physical delicate process, requiring precision to ensure that the tissue is successfully collected without contamination. No flow should be present in order to avoid the escape of the scrapped tissue.

The extraction of genomic DNA was performed using QIAamp DNA FFPE Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The protocol provided by the manufacturer was followed meticulously, using filtered pipette

tips. The entire process of DNA extraction was performed in a dedicated workstation in order to maintain contamination-free environment.

After extraction the samples were stored at -20ºC.

4.3.3.3 - HPV genotyping

For HR-HPV genotyping the Anyplex™ II HPV HR Detection kit (Seegene®, Seoul, Korea, acquired to Werfen, Portugal) was used, according to the manufacturer's instructions [146]. A multiplex real-time PCR (CFX96 PCR from Bio-Rad, USA) was performed. The kit enables the simultaneous genotyping of 14 distinct HPV types, including 12 high-risk types identified as Group 1 carcinogens (HPV-16/18/31/33/35/39/45/51/52/56/58/59). Additionally, it encompasses HPV-66, classified under Group 2B as possibly carcinogenic, and HPV-68, classified under Group 2A as possibly carcinogenic [141, 150]. The Anyplex™ II HPV HR Detection kit provides an internal control for each sample and a positive and negative controls for each plate of the real-time PCR reaction. The meticulous system control system is pivotal for ensuring the accuracy and reliability of the PCR results, offering an added layer of validation to the genotyping process. By including these controls, the kit effectively minimizes the potential for false-positive or false-negative results, thus providing an higher degree of confidence.

To prevent cross-contamination the PCR mixture was prepared within a UV PCR cabinet. Following this step, the addition of DNA was carried out in a vertical laminar flow cabinet. This cabinet ensures a sterile airflow, safeguarding the integrity of the samples and the accuracy of the genotyping process.

For genotyping, filtered pipette tips were also used, also as dedicated pipettes, to avoid cross-contamination.

The analysis of PCR data was conducted using the Seegene Viewer™ (Seegene®) software, a properly tool, specifically designed to interpret the data generated by the multiplex real-time PCR. The internal controls of the kit and the algorithm of the software allows us to confirm the DNA amplification and the types of HR-HPV type(s) present in each HSIL.

4.3.4 - Results

In the present study, a total of 45 paraffin-embedded biopsy slides, each meticulously cut to a thickness of 10µm and encapsulating HSIL, underwent the process of genomic DNA extraction. Out of the initial 45 samples processed for genomic DNA extraction, the procedure was successfully completed for 44 samples. One sample proved challenging, and genomic DNA extraction was not feasible, there are several possibilities like lower amount of tissue, long-term storage, handling error. The sample was not considered for the study. Therefore, the 44 successfully extracted genomic DNA samples were then genotyped for HPV using the HR-HPV Detection kit.

Following the genotyping process, the collected data were analyzed using Seegene Viewer™ (Seegene®).

Though this analysis it was verified that the majority of the samples, 38 of 44 (quoting to 86,36%), tested positive for one or more HR-HPV types and 6 samples (13,63%) samples tested negative for HR-HPV types (Table 21).

HPV status	***n* (%)**
	44 (100)
HPV-positive	38 (86,36)
HPV-negative	6 (13,63)

Table/Tabela 21 - HPV status of the 44 samples included in the study.

In the analysis conducted in this study the genotyping results from HR-HPV detection reveal a diverse distribution of HPV types among the cases examined. In the data, complied and presented in Table 22, it can be verified that positivity for HPV-16 was of 21 in 38 HPV-positive samples (55,26%).

Furthermore, it were identified 8 cases (21,05%) positive for HPV-31.

HPV genotypes	*n* (%)
HPV-16	21 (55,26)
HPV-18	2 (5,26)
HPV-31	8 (21,05)
HPV-33	0
HPV-35	3 (7,89)
HPV-39	2 (5,26)
HPV-45	0
HPV-51	1 (2,63)
HPV-52	4 (10,53)
HPV-56	2 (5,26)
HPV-58	2 (5,26)
HPV-59	2 (5,26)
HPV-66	1 (2,63)
HPV-68	0

Table/Tabela 22 - HR-HPV genotyping

HPV-52 and HPV-35 were detected in 4 (10,53%) and 3 (7,89%) cases, respectively. The analysis also revealed a lower frequency of HPV-18, 39, 56, 58, and 59, with each of these types found in 2 cases (5,26%). Lastly, the least common types detected in this cohort were HPV-51 and HPV-66, with each found in only one case (2,63%).

In the present study a significant finding was the identification of multiple HR-HPV infections, observed in 9 cases (23,68%). The detailed data regarding multiple co-infections is clarified and summarized in Table 23.

Co-infection	*n*
HPV-16 and HPV-18	2
HPV-16 and HPV-35	1
HPV-16 and HPV-59	1
HPV-16, HPV-31 and HPV-35	1
HPV-31 and HPV-39	2
HPV-35 and HPV-58	1
HPV-52 and HPV-56	1

Table/Tabela 23 - Co-infection of HPV genotypes

Among co-infections, double HR-HPV infections were the most commons, identified in 8 cases. Specifically, HPV-16 was involved in three distinct combinations of double infections: 2 cases were of co-infection with HPV-16 and HPV-18, and 1 case of each combination of HPV-16 with HPV-35 and of HPV-16 with HPV-59. Other double co-infection verified were 2 cases positive for HPV-31 and HPV-39. Moreover, individual cases of HPV-35/58 and of HPV-52/56.

Remarkably, it was also found a case of triple HR-HPV infection, positive for HPV-16, HPV-31 and HPV-35.

These results indicate that Anyplex™ is feasible for cervical lesions genotyping, it is also easy to perform and can be of great importance for risk stratification of high-grade cervical lesions, since the majority of the lesions were positive for HR-HPV and some lesions presented multiple HR-HPV infections.

4.3.5 - Discussion

Cervical cancer, a leading cause of cancer-related mortality among women worldwide, presents a substantial public health challenge [140]. Central to this issue is the role of persistent infections with oncogenic high-risk human papillomavirus, such as HPV-16 and 18. The HR-HPV types are etiologically linked to the majority of cervical cancer cases, making the detection an imperative for prevention.

The present study explores the need for early genotyping and monitoring of HSIL for high-risk HPV infections, which can lead to early intervention and potentially prevent the progression to cervical cancer.

HR-HPV types are present in the majority of cervical cancers, leading to high mortality rates. The current retrospective study was based on a cohort of 45 cases of paraffin-embedded samples of cervical lesions. Paraffin-embedded technique facilitates long-term storage of the biological specimens but the histological and molecular integrity is maintained. However, long-term storage and the thickness of the slides might be a limiting step for the use of the samples for DNA amplification and analysis.

Genotyping of HR-HPV types was performed for the first time in this type of tissue using the referred Anyplex™ II HPV HR Detection kit. Among the optimizations performed, like incubation periods for deparaffinization, the 10µm thickness of the slides was of main importance, in order to accomplish DNA integrity and concentration.

Although screening programs highly contributes to cervical cancer prevention, this methodology show us to be precise and able to detect HR-HPV types present in cervical lesions.

In the present work, in 44 from 45 cases (97,8%) it was possible to extract genomic DNA for multiplex real-time PCR amplification and HR-HPV detection. Among these 44 cases, 86,36% of the lesions tested positive for at least one HR-HPV type. The HPV-16 type was the most prevalent type (55,26%), which aligns with global epidemiological data, as a leading cause of cervical cancer, highlighting its significant oncogenic potential and the importance of its early detection [147; 148; 149].

The second most prevalent HR-HPV type was HPV-31, albeit less commonly implicated in cervical cancer when compared to HPV-16 and HPV-18 types it is also classified as high-risk oncogenic types [141]. The prevalence of HPV-31 is in accordance with the ones described in the literature for the European Continent [124].

After, the most common HPV types were HPV-52 and 35 (10,53% and 7,89%, respectively). The prevalence of HPV-52 was similar with the one found by Sousa et al., 2019 in population of the northern region of Portugal [150]. However, HPV-35 was present in higher number of cases in the present cohort when compared to the study in the northern region of Portugal, and is closer to the prevalence of HPV-35 in the Africa Continent [124, 150]. The cases studied revealed a lower frequency of HPV-18, 39, 56,

58, and 59, with each of these types found in 2 cases. The less frequent types were HPV-51 and HPV-66, with each found in only one case. The prevalence of these high-risk types are similar to the ones identified for the European Continent [124].

Despite the prevalence of each high-risk type, the data indicates the need of a more accurate approach taking into account the types of HPV. The methodology presented provides information on the types of HPV that lead to precancerous lesions, going beyond a screening program that detects the prevalence of infection.

Moreover, it was also verified the multiple co-infection that underscores the complex interaction of the different HPV genotypes in the pathogenesis of cervical lesions and potentially impacts the progression and management of the disease.

Almost a quarter of the lesions presented HR-HPV multiple infections (23,8%), similar results were found by Sousa et al. where 25,7% of the liquid-based cytology samples also have multiple infections [150]. The HR-HPV 16 was the more common type in co-infections, and there was 1 case of triple co-infection (HPV-16/35/59), which illustrates the diversity of HPV interactions that can occur within the cervical epithelium, probably leading to a more heterogenic behavior of HSIL, depending upon the types of HR-HPV present. This scenario highlights the potential for multiple high-risk HPV types to concurrently infect and influence the pathological landscape of cervical tissues. The presence of multiple HR-HPV genotypes in a single case raises important questions about the interactions between different HPV types and their collective impact in the severity, progression, and treatment response of cervical lesions.

This is the first time, to our knowledge, that paraffin-embedded slides of cervical lesions are genotyped using the Anyplex™ II HPV HR Detection kit and represents a significant methodological advancement, offering a more streamlined and efficient approach for earlier detection of the lesions precursors of cervical cancer development. The kit used in the present work, was so far used for liquid cytology specimens biopsies and now shows to be also an effective tool to be used in paraffin-embedded tissues.

Traditional methods for HPV detection often involve time-consuming and labor-intensive techniques. Using the referred kit for HPV detection in paraffin-embedded slides can provide a more efficient and accurate diagnostic tool. This improved accuracy is vital in ensuring that individuals at risk receive appropriate follow-up and treatment. The use of paraffin-embedded tissue slides is a common practice in pathology labs, as they allow for long-term storage of tissue samples.

Detecting high-risk HPV in cervical lesions has clinical relevance, giving the accurate information if lesion is due to HR-HPV and of which type. Therefore, it can aid clinicians in identifying patients who may require closer monitoring, and more accurate treatment.

This research contributes to the ongoing efforts in cervical cancer screening and prevention. By developing a more efficient and reliable method for detecting high-risk HPV, this study may have implications for public health programs and policies aimed at reducing the burden of cervical cancer, potentially reducing the need for unnecessary interventions and minimizing healthcare costs.

Ultimately, the clinical interest of this study lies in its potential to improve patient outcomes. Early detection of high-risk HPV can lead to timely interventions, which can significantly impact the prognosis and quality of life for individuals with pre-cancerous lesions.

In summary, high-risk HPV detection using the Anyplex™ II HPV HR Detection kit in paraffin-embedded slides from cervical lesions addresses a pressing clinical need in cervical cancer prevention and management. The study not only addresses a crucial gap in the literature but also lays groundwork for future research, particularly for risk stratifications and the development of early therapeutic approaches taking into consideration the HR-HPV type(s) identified in the lesion. The methodology purposed has the potential to enhance diagnostic accuracy, inform clinical decision-making, and contribute to broader public health initiatives focused on reducing the incidence and impact of cervical cancer.

We believe that this investigation opens a new window to future stratification of HSIL risk and therapeutic management. It advocates for expanded laboratory and clinical studies to further validate de protocol's effectiveness and explore its integration into cervical screening guidelines, ultimately aiming to mitigate the burden of this disease on a global scale.

Author Contributions

Conceptualization, L.B., A.C.R. and J.F-M.; methodology, M.A., V.C., D.C., L.S., C.S., P.P., S.C., J.V.; software, M.A. and P.P.; validation, L.B., Y.Y. and J.F-M.; formal analysis, M.A., V.C.; investigation, M.A., V.C.; resources, P.P., S.C., J.V., A.C.R., J. F-M., L.B.; data curation, M.A. and V.C.; writing—original draft preparation, M.A., V.C.; writing—review and editing, M.A., V.C., D.C., L.S., C.S., P.P., S.C., J.V., A.C.R., J.F-M. and L.B.;

supervision, A.C.R., J.F-M. and L.B.; project administration, A.C.R., J.F-M. and L.B.; funding acquisition, L.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of BEIRA INTERIOR UNIVERSITY (protocol code CE-UBI-Pj-2017-027.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Not applicable.

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Conflicts of Interest

The authors declare no conflict of interest.

Capítulo 5 – Discussão e perspetivas futuras

Foi intenção desta investigação contribuir para uma melhor assistência às mulheres que fazem prevenção do CCU fora do rastreio organizado, especialmente no âmbito das consultas de Ginecologia. Há um número importante de mulheres que fazem prevenção secundária fora do rastreio organizado e os estudos que estão na base deste trabalho foram realizados a partir de uma base de dados hospitalares, não integrados em programas de rastreio organizado, pelo que consideramos os nossos dados adequados para se poderem tirar algumas conclusões.

Com esta tese procurámos chamar a atenção e trazer a público uma questão médica, pouco abordada, negligenciada e mesmo pouco consensual na comunidade médica, mas muito praticada em Portugal, assim como em outros países, que é o rastreio oportunístico ou de conveniência do CCU, praticado especialmente nos consultórios dos médicos especialistas em Ginecologia e Obstetrícia.

Até onde é que este tipo de rastreio deve seguir as mesmas regras definidas para o rastreio organizado do CCU? Que problemas enfrenta? Na impossibilidade de fazermos uma avaliação exaustiva, centrámo-nos em 3 problemas que sentimos serem os que afetam todos os especialistas de Ginecologia e Obstetrícia: 1) o que fazer nos casos de teste de HPV positivo repetido, mas com citologia reflexa NILM e com lesão mínima? 2) será necessário solicitar citologia reflexa face a um teste de HPV 16 e/ou 18 positivo? 3) podemos tornar menos agressiva a nossa conduta face às lesões cervicais de HSIL?

Conduzimos o nosso estudo com base no programa de Rastreio do Centro Hospitalar Universitário da Cova da Beira, que pelas suas características, constitui uma boa aproximação do que se passa no Rastreio Oportunista que se pratica habitualmente: 1) dirigido a todas as mulheres sem rastreio recente; 2) efetuado por Ginecologista, após visualização crítica do colo do útero, com possibilidade, por exemplo, de protelar o rastreio na presença de vaginite, ou efetuar de imediato a colposcopia face a queixas de coitorragias, ou à constatação de lesão macroscopicamente não tranquilizadora; 3) possibilidade de correlacionar a história clínica da mulher com a prática do Rastreio do CCU.

No estudo levado a cabo no Centro Hospitalar Universitário da Cova da Beira, para avaliar da necessidade de realização (ou não) de citologia reflexa, em mulheres com testes de HPV positivos, repetidos, devemos salientar alguns aspetos a considerar:

1) O protocolo utilizado foi o protocolo de rastreio oportunístico (ou de conveniência) do CCU do departamento de Ginecologia do Centro Hospitalar Universitário da Cova da Beira, aberto a todas as pacientes que frequentaram aquelas consultas (incluindo grávidas e mulheres com menos de 30 anos) e que teve como principal objetivo, oferecer rastreio do CCU a todas as mulheres que recorriam ao Serviço de Ginecologia/Obstetrícia, e que não tinham sido submetidas a rastreio nos últimos dois anos.

2) Neste programa, o método de deteção primária de HPV foi o teste de Cobas® 4800 HPV, com citologia (co-teste) para os HPV positivos, que na altura não era utilizado no programa de rastreio organizado do CCU da Zona Centro do país, mas que a evidência científica já mostrava ser superior a sua realização pelo que houve a preocupação de dar um "passo em frente", no sentido de que uma melhor estratificação do risco de CIN3+ aumentasse a sensibilidade para a deteção precoce do CCU, com um valor preditivo negativo muito próximo de 100% [125]. A periodicidade adotada no rastreio foi de 3 anos, porque também, na altura em que foi implementado, a evidência científica apontava para os 3 anos, como sendo a frequência mais conveniente. O meio líquido para transporte e preservação das amostras para citologia, adotado para o programa de rastreio foi o Surepath®, por não apresentar diferenças significativas em termos de valor de corte quando comparado com outros meios de colheita certificados, e por na altura, ser considerado menos dispendioso e perfeitamente adequado à deteção do HPV, pelo equipamento COBAS utilizado.

3) O programa de rastreio do CCU instituído no Centro Hospitalar Universitário da Cova da Beira, iniciava-se aos 25 anos, para estar de acordo com o programa de rastreio do CCU da Zona Centro e, também porque na altura não existia a evidência científica que hoje existe, sobre a alta prevalência da infeção pelo HPV até aos 30 anos, o que tira especificidade ao rastreio. O estudo para além de se basear num programa de rotina institucional, baseado numa população aleatória que frequenta consultas ginecológicas (incluindo grávidas e mulheres com menos de 30 anos) tem também a limitação do tamanho da amostra, devido à baixa densidade populacional da Beira Interior. Além disso, algumas mulheres com teste de HPV positivo, não realizaram qualquer follow-up, por terem sido submetidas a intervenção cirúrgica para correção de uma condição

benigna (fibromiomas e/ou prolapso dos órgãos pélvicos) ou por terem abandonado as Consultas de Ginecologia do CHUCB.

4) A influência da vacinação contra o HPV nos resultados não foi avaliada por que a percentagem de mulheres vacinadas era pequena no momento da recolha de dados, e as que existiam tinham efetuado vacinação oportunista, em geral, já após o início da atividade sexual.

No entanto, os resultados do nosso estudo sobre a prevalência de anomalias citológicas e de HPV estão de acordo com as publicações de outros estudos realizados em diferentes países [89][119], pelo que podemos concluir que a população estudada foi adequada para extrair conclusões válidas. Além disso, os nossos resultados também estão de acordo com as diretrizes de Consenso de Gestão Baseada em Risco da ASCCP de 2019, publicadas recentemente [122], que recomendam a realização de colposcopias para todas as mulheres com testes positivos repetidos de HPV com NILM ou alterações citológicas minor.

Apesar das limitações já referidas, as ilações que retiramos deste estudo são corroboradas pelos estudos realizados em outros países, como o grupo de estudo HEllenic Real life Multicentric cErvical Screening, na Grécia (HERMES) [121] e um estudo que avalia os resultados de eficácia do teste primário de HPV com base no acompanhamento de ensaios clínicos randomizados na Alemanha (WOLPHSCREEN), Suécia (SWEDESCREEN), Inglaterra (ARTISTIC), Holanda (POBASCAM) e Itália (NTCC) [89].

O facto de os resultados do rastreio do CCU serem altamente dependentes do número de lesões detetadas pelas biópsias do colo do útero dirigidas por colposcopia, reforça a importância de práticas de colposcopia de qualidade.

No segundo estudo clínico, de acordo com o protocolo de rastreio oportunístico implementado no CHUCB já referido e com as limitações atrás enunciadas, quisemos avaliar se a citologia reflexa (co-teste) seria ou não útil para estratificar as mulheres com teste positivo de HPV 16 e/ou 18, em grupos de baixo e alto risco. Os resultados desse estudo apontavam no sentido de a citologia reflexa não parecer ser útil para o encaminhamento imediato para a colposcopia das mulheres com testes positivos de HPV 16 e/ou 18, já que a maioria delas com diagnóstico de HSIL+ tinha citologias reflexas com alterações menores que HSIL.

O protocolo de rastreio do CCU instituído no Centro Hospitalar Universitário da Cova da Beira considerou inicialmente a citologia reflexa nas mulheres que testavam positivo para o HPV 16 e 18, porque na altura em que foi desenhado ainda não existia evidência científica robusta, como há hoje, que permitisse dispensar aquela atitude. Foi assim considerado mais prudente fazer colpocitologia a todas as mulheres que testavam positivas para o HPV 16 e 18.

Na maioria dos estudos publicados sobre este tópico, parece evidente haver um risco aumentado de lesões histológicas HSIL+ em casos positivos para HPV16, em comparação com casos positivos para HPV18. No nosso estudo, essa diferença não pareceu significativa, possivelmente devido ao pequeno tamanho da amostra e às características da sua população. O risco de desenvolver uma lesão HSIL após um teste positivo para HPV 16 e/ou 18 é superior a 4%, pelo que, de acordo com as Diretrizes da ASCCP de 2019 [122], a referenciação imediata para colposcopia é sempre recomendada, sem necessidade de citologia reflexa, comportando um esforço considerável aos serviços de ginecologia, no âmbito do programa de rastreio.

Este estudo aponta para que, mesmo em consultas de Ginecologia em que o operador tem experiência na observação macroscópica do colo do útero e fácil acessibilidade ao uso do colposcópio, perante um teste de HPV 16 e/ou 18 positivo a citologia reflexa não traz benefício adicional. Nesse sentido, no âmbito das consultas de Ginecologia, parece fazer pouco sentido o uso do co-teste.

No que diz respeito à preocupação que esteve subjacente no nosso estudo laboratorial, para a deteção do tipo de HPV em material parafinado, esta incidiu sobre a possibilidade da rentabilização das atitudes terapêuticas face às lesões de HSIL do colo do útero. Os principais fatores de risco para o CCU são a idade da mulher e o tipo de HPV presente na lesão. Atualmente, a identificação do tipo de HPV responsável pela lesão de HSIL baseia-se na deteção do vírus em amostras de fluidos cérvicovaginais, método que nem sempre reflete com precisão a realidade, como demonstrado neste estudo.

Acreditamos que um dos principais obstáculos à utilização da deteção do tipo de HPV em material parafinado na prática clínica é a dificuldade técnica de remoção do DNA viral, o que tem limitado a realização de estudos nesta área. O nosso objetivo foi ir um pouco “mais além” destas limitações e proporcionar uma técnica sensível, acessível e de fácil execução, capaz de identificar com precisão o HPV envolvido no processo de carcinogénese de cada lesão HSIL do colo do útero.

Consideramos este estudo como piloto, com escassa casuística, pelo que entendemos ser de interesse e promissor a elaboração de um estudo mais alargado, preferencialmente multiinstitucional, não só para avaliar a validade da técnica que propomos, mas também da facilidade na sua execução.

O presente estudo explora a possibilidade de através de uma determinação precisa de genótipos de HPV e monitorização das HSIL por infeções por HPV-hr, poder levar a uma intervenção mais precoce e assim, potencialmente prevenir a progressão para CCU.

A técnica de embebição em parafina facilita o armazenamento a longo prazo dos espécimes biológicos, mantendo a integridade histológica e molecular. No entanto, o armazenamento a longo prazo e a espessura das lâminas podem ser uma etapa limitante para a utilização das amostras para amplificação e análise de DNA.

Embora os programas de rastreio contribuam significativamente para a prevenção do CCU, esta metodologia revela-se precisa e capaz de detetar os tipos de HPV-hr presentes nas lesões cervicais. No nosso estudo, em 44 dos 45 casos (97,8%) foi possível extrair DNA genómico para amplificação por PCR multiplex em tempo real e deteção de HPV-hr. Entre estes 44 casos, 86,36% das lesões HSIL apresentaram um resultado positivo para pelo menos um tipo de HPV-hr. O tipo HPV 16 foi o mais prevalente (55,26%), o que está de acordo com os dados epidemiológicos globais, como uma das principais causas de cancro do colo do útero, realçando o seu significativo potencial oncogénico e a importância da sua deteção precoce [16].

O segundo tipo de HPV-hr mais prevalente foi o HPV 31, embora menos comummente implicado no CCU, quando comparado com os tipos HPV 16 e HPV 18, é também classificado como tipo oncogénico de alto risco [12]. A prevalência do HPV 31 está de acordo com as descritas na literatura para o continente europeu [16].

Posteriormente, os tipos de HPV-hr mais comuns, foram o HPV 52 e o HPV 35 (10,53% e 7,89%, respetivamente). A prevalência do HPV 52 foi semelhante à encontrada por Sousa et al. (2019) [150] na população da região norte de Portugal. No entanto, o HPV 35 esteve presente num maior número de casos na presente coorte, quando comparado com o estudo realizado na região norte de Portugal, e aproxima-se da prevalência do HPV 35 no continente africano [16]. Os casos estudados revelaram uma menor frequência dos HPV 18, 39, 56, 58 e 59, tendo cada um destes tipos sido encontrado em 2 casos. Os tipos menos frequentes foram o HPV 51 e o HPV 66, cada um encontrado em apenas um caso.

A prevalência destes tipos de alto risco é semelhante à identificada para o continente europeu [16].

Apesar da prevalência de cada tipo de alto risco, os dados indicam a necessidade de uma abordagem mais precisa, tendo em conta os tipos de HPV. A metodologia apresentada fornece informação sobre os tipos de HPV-hr que levam a lesões pré-cancerosas, indo além de um programa de rastreio que deteta a prevalência da infeção.

Além disso, foi também verificada a coinfecção múltipla, que sublinha a complexa interação dos diferentes genótipos de HPV-hr na patogénese das lesões cervicais e potencialmente impacta a progressão e gestão da doença.

Quase um quarto das lesões apresentava infeções múltiplas por HPV-hr (23,8%), resultados semelhantes foram encontrados por Sousa et al. (2019) [150] onde 25,7% das amostras de citologia em meio líquido também apresentavam infeções múltiplas. O HPV 16 foi o tipo mais comum nas coinfecções, tendo havido 1 caso de tripla coinfecção (HPV 16/35/59), o que ilustra a diversidade de interações do HPV que podem ocorrer no epitélio cervical levando provavelmente a um comportamento mais heterogéneo da lesão HSIL, dependendo dos tipos de HPV-hr presentes. Este cenário realça o potencial de múltiplos tipos de HPV-hr infetarem e influenciarem simultaneamente o panorama patológico dos tecidos cervicais. A presença de múltiplos genótipos de HPV-hr num único caso, levanta questões importantes sobre as interações entre os diferentes tipos de HPV e o seu impacto coletivo na gravidade, progressão e resposta ao tratamento das lesões cervicais. Esta é a primeira vez, tanto quanto sabemos, que se procede à determinação dos genótipos de HPV, em lâminas de lesões cervicais embebidas em parafina, através do kit Anyplex™ II HPV HR Detection, o que representa um avanço metodológico significativo, oferecendo uma abordagem mais ágil e eficiente para a deteção precoce de lesões precursoras do desenvolvimento do CCU. O kit utilizado no presente trabalho, até então utilizado para biópsias de espécimes de citologia líquida, demonstra agora ser uma ferramenta eficaz também para utilização em tecidos embebidos em parafina.

Os métodos tradicionais de deteção do HPV envolvem frequentemente técnicas demoradas e trabalhosas. A utilização do referido kit para a deteção de HPV em lâminas embebidas em parafina pode fornecer uma ferramenta de diagnóstico mais eficiente e precisa, orientando para que os indivíduos em risco recebam o acompanhamento e o tratamento adequados. A utilização de lâminas de tecido embebidas em parafina é uma

prática comum nos laboratórios de patologia, uma vez que permitem o armazenamento de amostras de tecido a longo prazo. A deteção de HPV-hr em lesões cervicais tem relevância clínica, fornecendo informações precisas sobre se a lesão é causada por HPV-hr, de que genótipo, facilitando a identificação de doentes que possam necessitar de uma monitorização mais rigorosa e de um tratamento mais preciso, para além de monitorizar a eficácia da vacina, no caso de mulheres vacinadas.

Esta investigação contribui para os esforços contínuos de rastreio e prevenção do CCU. Ao desenvolver um método mais eficiente e fiável para a deteção de HPV de alto risco, este estudo pode ter implicações para os programas e políticas de saúde pública que visam reduzir a carga do CCU, reduzindo potencialmente a necessidade de intervenções desnecessárias e minimizando os custos com os cuidados de saúde.

Em última análise, o interesse clínico deste estudo reside no seu potencial para melhorar os resultados, em que a deteção precoce do HPV de alto risco pode levar a intervenções atempadas, o que pode ter um impacto significativo no prognóstico e na qualidade de vida dos indivíduos com lesões pré-cancerosas.

Em resumo, a deteção de HPV-hr, utilizando o kit Anyplex™ II HPV HR Detection em lâminas embebidas em parafina a partir de lesões cervicais, vem ao encontro de uma necessidade clínica na prevenção e tratamento do CCU.

O estudo não só aborda uma lacuna crucial na literatura, como também estabelece bases para futuras pesquisas, particularmente para estratificações de risco e o desenvolvimento de abordagens terapêuticas precoces, tendo em conta o(s) tipo(s) de HPV-hr identificado(s) na lesão. A metodologia proposta tem o potencial de aumentar a precisão diagnóstica, informar a tomada de decisões clínicas e contribuir para iniciativas mais amplas de saúde pública focadas na redução da incidência e do impacto do cancro do colo do útero.

Esta investigação deixa em aberto a possibilidade do alargamento dos estudos laboratoriais e clínicos para validar ainda mais a eficácia do protocolo e explorar a sua integração nas guidelines de rastreio cervical, visando, em última análise, mitigar a carga desta doença à escala global.

Consideramos que este estudo é um estudo piloto, com escassa casuística, pelo que entendemos ser de interesse e promissor, a elaboração de um estudo mais alargado,

preferencialmente multiinstitucional, não só para avaliar a validade da técnica que propomos, mas também da facilidade na sua execução.

Considerações Finais

O Rastreio do Cancro de Colo do Útero (RCCU) oportunístico ou de conveniência está fortemente implantado em Portugal e contribui de forma significativa para a redução da mortalidade por CCU, como acontece nos Estados Unidos da América e em muitos países europeus.

Há muito pouca investigação sobre o Rastreio oportunístico do CCU, pelo que se levantam questões que necessitam de uma resposta científica para o futuro:

1) Validação científica do rastreio Oportunista do CCU como prática médica adequada na prevenção do CCU;
2) Integração do Rastreio Oportunístico do CCU no programa nacional de prevenção secundaria do CCU, revestindo-o de regras e de uma responsabilidade que até à data não tem existido;
3) Controlo de qualidade na aplicação do método de rastreio, assegurando que as normas de qualidade laboratorial já definidas por lei, sejam implementadas por todos as instituições privadas;
4) Controlo das práticas médicas associadas ao Rastreio Oportunístico do CCU, o que passará inevitavelmente pela elaboração de guidelines orientadoras dessas práticas;
5) Custo-efetividade no apoio às mulheres que preferem optar pelo Rastreio Oportunístico do CCU, no sentido de o tornar mais acessível e mais inclusivo.

Esperamos que com esta tese tenhamos contribuído para um melhor entendimento do rastreio Oportunístico do CCU e sua contribuição para a melhoria da prevenção secundário do CCU, assim como para a melhoria da sua utilização, tão necessária, em prol da saúde da mulher.

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Anexos

Anexo 1

Artigo: "Repeated Positive Cervical HPV Testing and Absent or Minor Cytology Abnormality at Pap Smear. What is the Next Step?"

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RESEARCH ARTICLE

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Repeated Positive Cervical HPV Testing and Absent or Minor Cytology Abnormality at Pap Smear. What is the Next Step?

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Abstract

Background: Human papillomavirus (HPV) screening has significantly reduced cervical cancer (CC) mortality. Women who consecutively test positive for high-risk HPV without and minor changes on reflex cytology (atypical squamous cells of undetermined significance [ASC-US] or low-grade squamous intraepithelial lesion [LSIL]) or dysplasia on cervical colposcopy-oriented biopsy are always referred to colposcopy. The aim of the present study was to assess whether this guidance is appropriate for COBAS HPV testing with reflex cytology. **Methods:** A cross-sectional, retrospective study was carried out in 5,227 women who underwent routine CC screening over a period of five years (2012-2017). All HPV tests were performed using Cobas®4800 HPV. The study included women attending gynecology appointments whose first HPV test was positive and who had any type of follow-up. Patients' HPV test results as well as cytology and biopsy findings obtained during the abovementioned period were analyzed. A descriptive and comparative statistical study was conducted using this data. **Results:** A total of 765 out of 6003 HPV tests performed in 5,227 women were positive, and 141 women who had a positive HPV test (with negative for intraepithelial lesion or malignancy [NILM] or inflammation, or ASC-US and LSIL cytology, but no lesions on colposcopy, or absence of dysplasia on histology) repeated the HPV test at least once. Of these 141 women, 6 were diagnosed with high-grade squamous intraepithelial lesion (HSIL) during the follow-up period. All cases of HSIL were diagnosed after the second HPV test. **Conclusion:** This study shows that, at cervical cancer screening, all women testing positive for HPV regardless of Pap smear result should be referred to colposcopy.

Keywords: Cervical cancer- HPV testing- cervical cancer screening- HSIL- CIN

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Introduction

According to the World Health Organization (WHO) statistics, around 15 to 20% of the diagnosed cancers are associated with viral infections. Human papillomavirus (HPV) is one of the viruses contributing to these statistics, increasing the risk of cervical cancer (CC) progression when high-risk HPV infection persists (Chan et al., 2019). CC is the fourth most common cancer in women worldwide, after breast cancer, colorectal cancer and lung cancer (Bhatla and Denny, 2018).

Screening programs which incorporate HPV testing have consistently been associated with a reduction in CC incidence, potentially decreasing morbidity and mortality (Chan et al., 2019). Nevertheless, CC remains a major public health problem, with estimated 569,847 new cases and 311,365 deaths worldwide in 2018 (Bray et al., 2018).

Persistent infection with high-risk HPV genotypes is a necessary but not sufficient condition for disease progression and is the main epidemiological driver of high-grade intraepithelial lesions (HSIL) and invasive carcinoma (Oliveira et al., 2013). HPV infection is subclinical in most cases, especially in younger women where in more than 80% of cases the infection resolves spontaneously within 1 to 2 years. However, approximately 10% of HPV infections can become persistent and about 3 to 4% progress to intraepithelial lesions. Of these, 0.7 to 1% may advance to high-grade lesions (CIN 2/3), being estimated that 0.1% will progress to invasive cancer if not detected and treated in a timely manner (WHO, 2012).

The natural course of CC is well known, and its carcinogenesis process is slow. The presence of CC precursor lesions, the availability of sensitive screening tests for detection and effective treatment methods have enabled highly effective secondary prevention, using screening programs (Tsikouras et al., 2016).

The most common CC screening methods are conventional cytology, liquid-based cytology and HPV testing, or an association of the latter two (WHO, 2012). The "standard" screening method has been morphological

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cytology. Several studies have shown that HPV testing is more sensitive than cytology alone in detecting and preventing high-grade lesions and progression to cancer. In addition, when using HPV testing as a screening method, the presence of a negative test allows the screening interval to be extended to 5 years, improving compliance with screening programs and enabling effective cost reductions of approximately 20% (Schiffman et al., 2011; Agorastos et al., 2015; Goodman, 2015; Tsikouras et al., 2016).

In 2017, a national organized CC HPV-based screening program was implemented in Portugal for women between the ages of 25 and 60 years, performed every 5 years, with reflex cytology for high-risk HPV genotypes other than HPV 16 and 18. This screening program introduces updates to the previous regional cervical cancer screening programs and states that women with a positive HPV test for genotypes other than 16 and 18 with negative for intraepithelial lesion or malignancy (NILM) cytology should repeat the HPV test within the following year. In case of a second HPV test is positive, the woman will be referred for colposcopy. Following 2013 Kaiser Permanent Northern California (KPNC) study results, women with a repetitive positive HPV test with without or minor cytological abnormality (ASC-US/LSIL) and no dysplasia on cervical oriented-colposcopy biopsy should be recommended to colposcopy based on co-testing and Hybrid Capture 2 (HC2; Qliagen, Germantown, MD) for HPV testing (Katki et al., 2013). However, no scientific report showed whether this approach is useful on cervical cancer screenings based on primary new molecular technologies for HPV testing with reflex cytology.

Our goal was to use the opportunistic CC screening program of the Cova da Beira University Hospital Center (CHUCB), based on primary COBAS HPV testing and triage cytology, to validate colposcopy recommendation for those women with repeated positive HPV test for genotypes other than HPV 16 and 18 and NILM or minor lesions on previous cytology and no previous dysplasia detected on cervical oriented-colposcopy biopsy.

Materials and Methods

A cross-sectional and retrospective study was carried out based on data from the routine CC screening protocol in force at CHUCB between August 2012 and August 2017. The screening protocol was based on HPV testing as the primary method for all women over 25 years old with no history of CC screening in the past 2 years who attend gynecology appointments at the CHUCB.

The screening method was the Cobas®4800 HPV test, which detects HPV 16, HPV 18 and other types of HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68), using the liquid medium Surepath®.

All HPV tests were performed at the Laboratory of the Clinical Pathology Department of the CHUCB, while the cytological and histological tests were conducted at the Anatomical Pathology Department of the CHUCB.

The CC screening program of the CHUCB was designed and implemented by the CHUCB Colposcopy Unit, where all colposcopic examination were performed. According to the CHUCB screening algorithm, shown in Figure 1, a negative HPV tests should be repeated after 3 years; a positive HPV test for genotypes 16 or 18 is followed by reflex cytology and referral for a colposcopy; a positive HPV test for other types of HPV is followed by reflex cytology, and if reflex cytology shows NILM, the test must be repeated after 1 year; any other cytological finding requires referral for a colposcopy. After a second consecutive positive HPV test, the woman is monitored at the Colposcopy Unit for at least 3 years, regardless of subsequent HPV test results and cytology findings.

Of all 6,003 HPV tests performed in 5,227 women who underwent routine screening at the CHUCB over the abovementioned 5 years, 765 (14.6%) women who had a positive HPV test were selected for our study. Of these, we evaluated 141 women who had no history of treatment for cervical intraepithelial neoplasia and had satisfactory cytology findings, classified as NILM or minor cytological lesions (ASC-US/LSIL), normal colposcopy and/or no dysplasia on biopsy, and who had follow-up appointments at the Colposcopy Unit of the CHUCB. A biopsy was required during a colposcopy appointment only in the presence of grade 1 or 2 colposcopic findings or signs of invasion. If the transformation zone is classified as type 3 (squamouscolumnar junction not fully visible), endocervical curettage is performed routinely.

A descriptive statistical analysis of the data was performed, using the IBM SPSS application software, version 26 (SPSS Inc., Chicago, IL). In all cases, we analyzed the patient's age, HPV test results (type 16, 18 and others), and cytology and histology findings of the biopsy obtained using colposcopy.

Results

The study sample consisted of 141 women who had a positive HPV test (to HPV 16, 18 and others) with reflex cytology classified as NILM or minor cytological lesions (ASC-US/LSIL), but normal colposcopy and/or no dysplasia on biopsy and who underwent follow-up (Table 1). This corresponds to 18.4% of all women with a positive HPV test during the study, aged between 17 and 69, with a mean age of 39.3 years (standard deviation=11.1). For these women, the mean follow-up was 36.6 months (standard deviation=18.5).

During follow-up, CIN2+ lesions were detected in six (4.3%) women, with a mean age of 35.7 years (standard deviation= 7.7), and all CIN2+ lesions were diagnosed after the second HPV test. No women were diagnosed with invasive carcinoma. The mean time to diagnosis of CIN2+ lesions was 18.5 months (standard deviation=4.2). The HPV test, cervical cytology and biopsy results are shown in Table 1. Regression rate of HPV infection in the studied group was always very high, especially for types 16 and 18, which highlights the transient nature of those HPV infections. However, the multiple infection rate (HPV 16 or 18 and others) remained unchanged, possibly due to reinfection. Following the first test, only 45 women underwent colposcopy due to a positive HPV 16 or 18 test and/or ASC-US or LSIL cytology. All women underwent colposcopy in their second, third, fourth and fifth HPV tests. Six women were co-tested for their second HPV test,

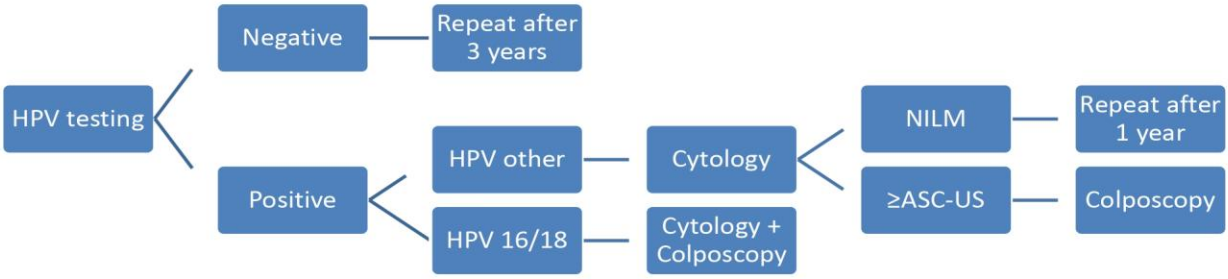

Figure 1. Flowchart of Cervical Cancer Screening Implemented on Cova da Beira University Hospital Center (CHUCB). A negative HPV test should be repeated after 3 years. However, women with HPV positive test for other strains than 16 or 18 are examined by reflex cytology. In case of negative for intraepithelial lesion or malignancy (NLIM), cytology should be repeated after one year. Otherwise, women should be referred to colposcopy. In case of infection by HPV 16 or 18 genotypes, the follow-up incorporates both cytology and colposcopy.

and 3 women were co-tested for their third and fourth tests.

Table 2 shows that the prevalence and spontaneous resolution of high-risk HPV infection was more common in women under 30 years of age, while the cytological and histological diagnosis was more serious in the group of women over 30 years of age.

Table 3 shows the relevant aspects of the 6 cases where HSIL was diagnosed during patient follow-up. Four of these 6 cases were diagnosed in women aged 30 or over. HSIL was associated with HPV 16 infection in only one woman, and cytology had been classified as NILM or ASC-US or LSIL in 4 women.

Discussion

The protocol used in this study was the CC screening protocol of the Gynecology Department of the CHUCB, which recommends HPV testing as the primary test in routine screening. This is an institutional screening program which, among other aspects, is open to all patients attending gynecology appointments (including pregnant women) and had the participation of all physicians who offer gynecology appointments at the CHUCB. This cervical cancer screening was implemented at the CHUCB to manage patients and to mitigate the effects of low compliance with the national screening program.

The CC screening protocol at the Gynecology Department of the CHUCB beginning at 2012 was organized following 2011 ATHENA HPV study results (Wright et al., 2012).

A high percentage of women under the age of 30 were included in the study population. The CC screening protocol in force at the CHUCB includes women over 25 years of age and some physicians did not comply with

Table 1. Sequency of Results Human Papillomavirus (HPV) Tests, Cytology and Histology Results. Data is presented as number (percentage, %).

	1st Test (n=141)	2nd Test (n=141)	3rd Test (n=55)	4th Test (n=19)	5th Test (n=6)
HPV test					
Negative	-	61 (43.3)	20 (36.4)	4 (21.1)	3 (50)
HPV 16	16 (11.3)	6 (4.2)	2 (3.6)	1 (5.3)	1 (16.6)
HPV 18	5 (3.5)	-	1 (1.8)	-	-
Others	102 (72)	59 (41.8)	26 (47.3)	11 (57.9)	2 (33.4)
HPV 16+others	15 (10.6)	11 (7.8)	5 (9.1)	2 (10.6)	-
HPV 18+others	3 (2.1)	3 (2.1)	1 (1.8)	1 (5.3)	-
CYTOLOGY					
Not performed	1 (0.7)	55(39)	17(31)	1(5.3)	3(50)
NILM	101 (71.7)	51 (36.2)	28 (50.9)	13 (68.4)	2 (33.3)
LSIL	24 (17.0)	13 (9.3)	3 (5.4)	2 (10.5)	-
ASC-US	15 (10.6)	15 (10.6)	6 (10.9)	2 (10.5)	1 (16.7)
HSIL	-	3 (2.1)	1 (1.8)	1 (5.3)	-
ASC-H	-	4 (2.8)	-	-	-
HISTOLOGY					
Not performed	24 (53.3)	104 (73.7)	49 (89.1)	14 (73.7)	4 (66.6)
No dysplasia	21 (46.7)	18 (12.8)	4 (7.3)	3 (15.8)	1 (16.7)
LSIL	-	13 (9.2)	2 (3.6)	2 (10.5)	1 (16.7)
HSIL	-	6 (4.3)	-	-	-

ASC-H, atypical squamous cells; ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

Table 2. Sequency of Human Papillomavirus (HPV) Tests, Cytology and Histology Results Comparing Women under and over 30 Years of Age. Data is presented as number (percentage, %).

	1st Test		2nd Test		3rd Test		4th Test		5th Test	
Age	<30	>30	<30	>30	<30	>30	<30	>30	<30	>30
Number of cases	30 (21.3)	111 (78.7)	30 (21.3)	111 (78.7)	11 (20)	44 (80)	2 (1.1)	17 (98.9)	-	6 (100)
HPV TEST										
Negative	-	-	15 (50)	46 (41.4)	4 (36.4)	16 (36.3)	1 (50)	3 (17.6)	-	3 (50)
HPV 16	8 (26.7)	8 (7.2)	1 (3.3)	5 (4.5)	1 (9.1)	1 (2.3)	-	1 (5.9)	-	1 (16.7)
HPV 18	-	5 (4.5)	-	-	1 (9.1)	-	-	-	-	-
Others	19 (63.3)	83 (74.8)	9 (30)	50 (45)	5 (45.4)	21 (47.7)	1 (50)	10 (58.8)	-	2 (33.3)
HPV 16+HPV 18	-	-	-	1 (0.9)	-	-	-	-	-	-
HPV 16+others	3 (10)	12 (10.1)	4 (13.3)	7 (6.3)	-	5 (11.4)	-	2 (11.8)	-	-
HPV 18+others	-	3 (2.7)	1 (3.3)	2 (1.8)	-	1 (2.3)	-	1 (5.9)	-	-
CYTOLOGY										
Not performed	1 (3.3)	-	14 (46.7)	41 (37)	3 (27.3)	14 (31.8)	1 (50)	-	-	3 (50)
NILM	20 (66.7)	81 (73)	12 (40)	39 (35	8 (72.7)	20 (45.5)	1 (50)	12 (70.6)	-	2 (33.3)
LSIL	6 (20)	18 (16.2)	2 (6.7)	11 (10)	-	3 (6.8)	-	2 (11.8)	-	-
ASC-US	3 (10)	12 (10.8)	1 (3.3)	14 (12.6)	-	6 (13.6)	-	2 (11.8)	-	1 (16.7)
HSIL	-	-	1 (3.3)	2 (1.8)	-	1 (2.3)	-	1 (5.8)	-	-
ASC-H	-	-	-	4 (3.6)	-	-	-	-	-	-
HISTOLOGY										
Not performed	6 (20)	18 (16.2)	24 (80)	80 (72)	9 (81.8)	40 (91)	1 (50)	12 (70.6)	-	4 (66.6)
No dysplasia	3 (10)	18 (16.2)	2 (6.7)	14 (12.6)	2 (18.2)	2 (4.5)	1 (50)	3 (17.6)	-	1 (16.7)
LSIL	-	-	-	13 (11.7)	-	2 (4.5)	-	2 (11.8)	-	1 (16.7)
HSIL	-	-	2 (6.7)	4 (3.6)	-	-	-	-	-	-

ASC-H, atypical squamous cells; ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy.

the inclusion criteria. There was a higher prevalence and spontaneous resolution of high-risk HPV infection in the group of women under 30 years of age, as well as less serious cytological and histological diagnoses, which is in accordance with the literature.

The HPV test used for screening was the Cobas®4800 HPV test, which is a qualitative test that uses real-time PCR technology to simultaneously detect DNA from 12 types of human recombinant HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and individually detect HPV 16 and 18. β-globin gene is amplified as an internal control. It can be used as a primary screening method with reflex cytology for positive HPV or in addition to cytology (co-testing). Thus, CIN3+ risk stratification is improved, increasing sensitivity for early detection of cervical cancer, with a negative predictive value very close to 100% (Chan et al., 2019).

The liquid medium used for transport and preservation of all samples for cytology was SurePath®, which does not exhibit significant differences in terms of cut-off values, when compared to other certified liquid collection media, for the detection of CIN1, CIN2+ and CC lesions (Rozemeijer et al., 2016).

Some women who had a positive HPV test did not undergo follow-up because they had a surgery for a benign condition (uterine fibroids or pelvic organ prolapse

Table 3. Detailed Description of High-Grade Squamous Intraepithelial Lesion (Cases of HSIL) Diagnosed during Study Follow-up.

	Description of positive cases					
Age	1st HPV Test	1st Cytology	2nd HPV Test	2nd Cytology	Time to diagnosis	Notes
25 years old	16 + Others	NILM	Others	HSIL	18 months	
27 years old	16 + Others	LSIL	16 + Others	LSIL	22 months	1)
30 years old	Others	NILM	Others	HSIL	25 months	
40 years old	Others	NILM	Others	NILM	16 months	2)
42 years old	Others	LSIL	Others	ASC-US	14 months	
50 years old	Others	LSIL	Negative	ASC-US	16 months	3)

ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; 1) Despite LSIL cytology, the patient underwent colposcopy and biopsy revealing HSIL; 2) Despite NILM cytology, the patient underwent colposcopy and biopsy revealing HSIL; 3) Co-testing (HPV testing + cytology) Patient underwent colposcopy and biopsy revealing HSIL

corrections), or they stopped attending appointments.

In accordance with literature, the percentage of multiple infections was different according to age, suggesting transient reinfection rather than a persistent infection (Pista et al., 2011).

From the analysis of the 6 cases of HSIL diagnosed, we highlight the importance of performing colposcopy after the second test, as all our cases were diagnosed at this time. Our results are validated by other studies reporting similar situations (Melnikow et al., 2018; Gu et al., 2019). The second cytology was suggestive of HSIL in only two cases, and it was classified as NILM in one case, which reinforces the value of colposcopy in these situations.

The absence of HSIL diagnosed after the second HPV test is probably due to the referral for colposcopy of all patients after the second positive HPV test, regardless of cytology findings. This procedure allowed HSIL identification which was not diagnosed during the first test. Between the first and second tests, it is more likely that there was regression than progression of dysplastic lesions, which may also explain in part why no other cases of HSIL were diagnosed after the second test. The outcome of any HPV-based CC screening is highly dependent on the number of lesions detected using colposcopy-directed biopsy, which reinforces the importance of quality colposcopy practices. Avoiding unnecessary biopsies without neglecting the diagnosis of cervical cancer precursor lesions is of paramount importance, and all women with positive HPV tests should be referred to different colposcopy units, as was the case in this study.

This study demonstrates that women undergoing HPV-based CC screening who had one positive HPV test with NILM, ASC-US or LSIL cytology, with normal colposcopic findings and/or no dysplasia on cervical biopsy, should be referred for colposcopy in the presence of a second positive HPV test, regardless of the cytology findings. This procedure is standardized in the current cervical cancer screening program in Portugal and recommended by 2019 American Society for Colposcopy and Cervical Pathology (ASCCP) guidelines (Perkins et al., 2020).

This study has some limitations. The CC screening method used at the CHUCB is an institutional routine program based on a random population that attends gynecology appointments and includes pregnant women, and it is not an organized screening program. Furthermore, sample size is limited as the geographic localization of CHUCB only serves a population of approximately 90,000 people which includes the municipalities of Covilhã, Fundão, Belmonte and Penamacor. Only women referred by CHUCB physicians to the CHUCB Colposcopy Unit were evaluated in this study. Many of these women had previously participated in the organized cervical cancer-screening program in the Centre Region of Portugal, which has been in place for more than 20 years. The influence of HPV vaccination on the results was not evaluated because the percentage of vaccinated women was small at the time of data collection.

Nevertheless, the results of our study concerning HPV and cytology abnormalities prevalence are in agreement with studies performed in other countries, such as the HEllenic Real life Multicentric cErvical Screening study group, in Greece (HERMES) (Agorastos et al., 2015) and a study that evaluates the efficacy outcomes of primary HPV testing based on the follow-up of randomized controlled trials in Germany (WOLPHSCREEN), Sweden (SWEDESCREEN), England (ARTISTIC), the Netherlands (POBASCAM) and Italy (NTCC) (Ronco et al., 2014). Therefore, we can conclude that our studied population was adequate for valid conclusions. In addition, our study is in agreement with recent published 2019 ASCCP Risk-Based Management Consensus Guidelines for Abnormal Cervical Cancer Screening Tests and Cancer Precursors (Perkins et al., 2020), that recommend colposcopies for all women with repeated positive HPV testing with NILM or minor cytology abnormalities.

This study has shown that, regardless of reflex cytology findings, women who have at least two consecutive positive cervical HPV tests are at increased risk of having previously undiagnosed cervical HSIL and should always be referred for colposcopy. Additionally, the risk of intraepithelial lesions or malignancy was independent of the type of HPV determined. All women with cervical repeated positive HPV testing and with absent or minor cytology abnormalities should be referred to colposcopy in an independent way of screening adopted program and technology used for HPV testing, as recommended by ASCCP.

Abbreviations

ASCCP – American Society for Colposcopy and Cervical Pathology; ASC-H – atypical squamous cells; ASC-US – atypical squamous cells of undetermined significance; CC – cervical cancer; CHUCB – Cova da Beira University Hospital Center; CIN – cervical intraepithelial neoplasia; Co-testing: Concomitant HPV test and cytology; HPV – human papillomavirus; HSIL – high-grade squamous intraepithelial lesion; KPNC – Kaiser Permanent Northern California; LSIL – low-grade squamous intraepithelial lesion; NILM – negative for intraepithelial lesion or malignancy; SPSS – Statistical Package for the Social Sciences.

Author Contribution Statement

Vitor Caeiro: Conducted the investigation and write manuscript; Sara Nunes: Organize statistical analysis of data and review the manuscript; Bruno Esteves: Responsible by HPV testing and pap smear and review the manuscript; José Fonseca-Moutinho; Advisor and review the manuscript.

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Conflict of interest

The authors have no conflict of interest to declare.

This research was approved Ethics Committee of Beira Interior University with the code CE-UBI-Pj-2017-027.

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Anexo 2

Artigo: "HPV testing for cervical cancer screening: Should reflex cytology be performed after a positive test for HPV 16 and 18?"

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HPV testing for cervical cancer screening: Should reflex cytology be performed after a positive test for HPV 16 and 18?

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ABSTRACT

At Portuguese-organized cervical cancer (CC) screening programs, all women testing positive for human papillomavirus (HPV) 16 and/or 18 are referred for immediate colposcopy. This study aimed to evaluate the utility of reflex cytology in women who test positive for HPV 16 and/or 18 to improve the efficiency of CC screening.

A cross-sectional and retrospective study was performed based on data from the routine CC screening protocol in force at Cova da Beira University Hospital Center, Portugal between August 2012 and June 2021. The screening method was the Cobas 4800 HPV test using the liquid medium Surepath. In all the selected cases, the patient's HPV test results and the cytology and histology findings of the biopsies obtained using colposcopy were analyzed.

This study included 339 women who first tested positive for HPV 16 and/or 18 and were referred for immediate colposcopy, in whom 40 (11.8%) cases of high-grade squamous intraepithelial lesion (HSIL+) were diagnosed. Of these, 12 (30%) had reflex cytology negative for intraepithelial lesion or malignancy (NILM) and 14 (35%) had HSIL+ cytology. After 3 years, 14 (9.3%) of the 150 women who were still undergoing follow-up were diagnosed with histologic HSIL+ lesions, of which 5 (35.7%) had baseline NILM cytology.

Despite the small sample, the results of this study allow us to conclude that reflex cytology is not useful for discrimination to immediate referral for colposcopy in women who test positive for HPV 16 and/or 18, as most women with a histologic diagnosis of an HSIL+ lesion had <HSIL reflex cytology.

Introduction

Cervical cancer (CC) is the fourth most common cancer in women worldwide, following breast cancer, colorectal cancer and lung cancer [1,2]. Persistent infection with high-risk HPV genotypes is the main epidemiological factor responsible for high-grade intraepithelial lesions (HSIL) and invasive carcinoma; however, it is not a determinant condition [3].

Early CC screening is very helpful in the management of disease severity, with conventional cytology, liquid-based cytology, and HPV testing, or a combination of the latter two, being the most used methods [4].

Detection and prevention of high-grade lesions are shown to be more effective with HPV testing rather than cytology alone. Moreover, HPV testing that has a negative result allows longer screening intervals (up to 5 years), improving compliance and reducing the associated cost by approximately 20% [5–8].

In 2017, a nationally organized CC HPV-based screening program was implemented in Portugal for women between the ages of 25 and 60 years, performed every 5 years, with reflex cytology for high-risk HPV genotypes other than HPV 16 and 18. This screening program introduces updates to the previous regional cervical cancer screening programs and states that women with a positive HPV test for genotypes other than 16 and 18 with cytology negative for intraepithelial lesion or malignancy (NILM) should repeat the HPV test within the following year. Immediate referral for colposcopy in women who test positive for HPV 16 and 18

List of abbreviations: ASC-H, atypical squamous cells; CC, cervical cancer; CHUCB, Cova da Beira University Hospital Center; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; NILM, negative for intraepithelial lesion or malignancy; SPSS, Statistical Package for the Social Sciences.

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was based on the findings of increased risk for HSIL+ lesions, evidenced in the ATHENA [9], ARTISTIC [10], and KPNC [11] studies. However, most women who test positive for HPV genotypes 16 and 18 and are immediately referred for colposcopy are not diagnosed with an HSIL+ lesion, meaning that they unnecessarily undergo a procedure with some morbidity.

In the KPNC and Athena studies, in cases where cotesting revealed a positive HPV test for genotypes 16 and 18 and HSIL cytology, the risk of immediate HSIL lesions was 40%, compared with approximately 10% in women with <HSIL cytology [9,11], which suggests that reflex cytology, in these cases, could inform colposcopy practice. In Portugal, as the national cervical cancer screening program does not provide for cytology (cotesting or reflex testing), the potential interest of performing reflex cytology in cases of positive HPV 16 and 18 testing has not yet been determined.

This study aims to evaluate the usefulness of reflex cytology in women who test positive for HPV 16 and/or 18, in order to improve the efficiency of Portuguese CC screening.

Material and methods

Study design and settings

A cross-sectional and retrospective study was carried out based on data from the routine CC screening protocol in force at Cova Beira Hospital Center (CHUCB) between August 2012 and June 2021. The study was reviewed and approved by the Ethics Committee of the University of Beira Interior and carried out in accordance with relevant guidelines and regulations.

CHUCB's catchment area includes the municipalities of Covilhã, Fundão, Belmonte, and Penamacor, and the hospital center provides care to a population of approximately 90,000, so the sample size was limited.

Participants

Data on all women who attended gynecology appointments at the CHUCB who were over 25 years old and had no history of CC screening in the past 2 years were included in this study.

Of all 8022 HPV tests performed on 6376 women who underwent routine screening at the CHUCB over the above-mentioned 9 years (August 2012 – June 2021), all 339 (5.3%) women who first tested positive for HPV genotypes 16 and/or 18 and underwent reflex cytology were selected for our study.

In all these cases, we analyzed the patient's HPV test results (type 16 and/or 18) and cytology and histology findings of the biopsies obtained using colposcopy (immediately and after 3 years).

For the data analysis, as there were only two cases of atypical squamous cells (ASC—H), these were included in the HSIL+ cytology.

Variables and data source

The screening protocol used in this study followed the routine screening algorithm implemented at CHUCB in 2012 (Fig. 1). Thus, all women with positive HPV-HR tests underwent reflex cytology, and those who tested positive for HPV genotypes 16 and 18 were also referred for colposcopy.

HPV testing was used as the primary method and performed with a Cobas 4800 HPV test, using the liquid medium Surepath. By using real-time PCR technology and the β-globin gene as an internal control for the test, this qualitative analysis is used to simultaneously detect DNA from 12 types of human recombinant HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and individually detect HPV 16 and 18. HPV testing can be used as a primary screening method with reflex cytology for positive HPV or in addition to cytology (cotesting). Previous studies showed that HPV testing improves CIN3+ risk stratification, which allows early detection of cervical cancer, with increased sensitivity and negative predictive values very close to 100% [12].

All HPV tests were performed at the laboratory of the Clinical Pathology Department of the CHUCB, while cytologic and histologic testing were conducted at the Anatomical Pathology Department of the CHUCB.

The CC screening program was designed and implemented by the CHUCB Colposcopy Unit, where all colposcopies were performed.

According to the CHUCB screening algorithm, shown in Fig. 1, a positive HPV test for genotypes 16 or 18 is followed by reflex cytology and referral for a colposcopy. Only patients with abnormal colposcopic findings underwent biopsy, while the remaining patients were instructed to undergo a repeat HPV test within one year.

Statistical analysis

Data were entered and analyzed using SPSS Statistics for Windows, Version 21.0 (Armonk, NY: IBM Corporation). Proportions, arithmetic means, medians, and standard deviations (SD) were used as summary statistics.

Results

The study sample consisted of 339 women who first tested positive for HPV genotypes 16 and/or 18 with reflex cytology (Table 1). In these women, all of whom underwent immediate colposcopy, 40 (11.8%) cases of HSIL+ lesion were diagnosed, of which 3 were invasive carcinomas (Table 2).

The mean age of the 6376 women included in the screening program was 43 years (standard deviation 13.03), and the mean age of the 339 women who tested positive for HPV genotypes 16 and/or 18 and were included in the study was 39.7 years (standard deviation 11.51).

Of the 279 women who tested positive for HPV genotype 16, 25 (9%) had HSIL+ cytology, and 254 (91%) had ≤HSIL+ cytology. Seventy-six

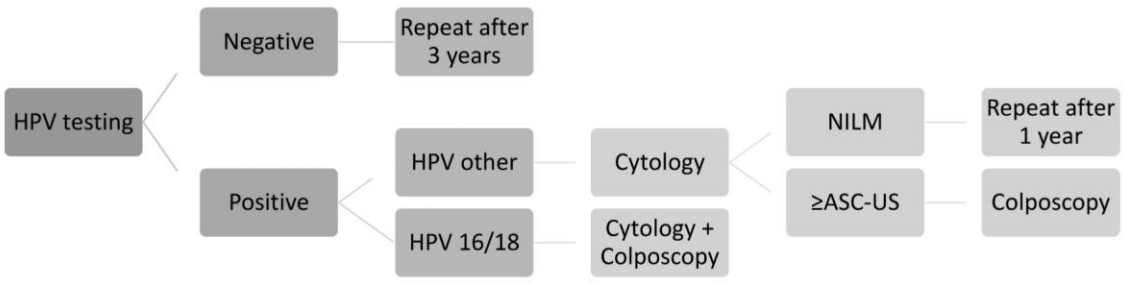

Fig. 1. . Flowchart of cervical cancer screening implemented on Cova da Beira University Hospital Center (CHUCB). A negative HPV test should be repeated after 3 years. However, women with HPV positive test for other strains than 16 or 18 are examined by reflex cytology. In case of negative for intraepithelial lesion or malignancy (NLIM), cytology should be repeated after one year. Otherwise, women should be referred to colposcopy. In case of infection by HPV 16 or 18 genotypes, the follow-up incorporates both cytology and colposcopy.

Table 1
HPV test and cytology results ($n = 339$).

HPV test		Baseline reflex cytology NILM	LSIL	ASC-US	HSIL+
HPV 16	163	106	25	16	16
HPV 18	28	18	4	2	4
HPV 16+HPVP	107	56	27	16	8
HPV 18+HPVP	32	23	6	3	–
HPV 16+18	5	3	2	–	–
HPV 16+18+HPVP	4	2	–	1	1
TOTAL	**339**	**208**	**64**	**38**	**29**

ASC-US – atypical squamous cells of undetermined significance; HPV – human papillomavirus; HSIL – high-grade squamous intraepithelial lesion; LSIL – low-grade squamous intraepithelial lesion; NILM – negative for intraepithelial lesion or malignancy.

Table 2
Baseline HPV test and cytology results ($n = 339$).

HPV test		Histology (immediate) Not performed	No dysplasia	LSIL	HSIL+
HPV 16	163	117	10	21	15
HPV 18	27	19	1	4	3
HPV 16+HPVP	107	78	2	11	16
HPV 18+HPVP	33	22	1	6	4
HPV 16+18	5	4	–	1	–
HPV 16+18+HPVP	4	2	–	–	2
Total	339	242	14	43	40

ASC-US – atypical squamous cells of undetermined significance; HPV – human papillomavirus; HSIL – high-grade squamous intraepithelial lesion; LSIL – low-grade squamous intraepithelial lesion; NILM – negative for intraepithelial lesion or malignancy.

of these 279 patients underwent colposcopy-directed biopsy, of whom 33 (11.8%) had histologic HSIL+ and the remaining 43 had histologic ≤HSIL+ (Tables 2 and 3).

Of the 60 women who tested positive for HPV genotype 18, 4 (7%) had HSIL+ cytology and 56 (93%) had ≤HSIL+ cytology. Nineteen (31.6%) of these 60 women underwent colposcopy-directed biopsy; 7 (11.7%) had histologic HSIL+ and the remaining 12 had histologic ≤HSIL+ (Tables 2 and 4).

After 3 years, 92 of the 242 women who did not undergo a biopsy at baseline were lost to follow-up due to hysterectomy for a benign condition, pregnancy, or drop-out. Fourteen (9.3%) of the 150 women who continued to undergo follow-up were diagnosed with histologic HSIL+ lesions during the following 3 years of follow-up. Of these 14 patients, 5 (35.7%) had baseline NILM cytology (Table 3).

Table 4 shows the histologic findings of the colposcopy-directed biopsies. We can see that 12 (30%) of the 40 patients diagnosed with HSIL+ had NILM reflex cytology and only 14 (35%) had HSIL+ cytology. Table 4(A) and 4(B) compile the colposcopic findings and histology results at baseline.

Table 3
Histology findings during 3 years of follow-up.

Baseline reflex cytology		Histology findings at 3 years Not performed	No dysplasia	LSIL	HSIL+
NILM	104	88	2	9	5
ASC-US	25	22	1	1	1
LSIL	17	11	–	1	5
HSIL+	4	1	–	–	3
Total	150	122	3	11	14

ASC-US – atypical squamous cells of undetermined significance; HPV – human papillomavirus; HSIL – high-grade squamous intraepithelial lesion; LSIL – low-grade squamous intraepithelial lesion; NILM – negative for intraepithelial lesion or malignancy.

Table 4
Baseline cytology and histology results.

Baseline reflex cytology		Baseline histology Not performed	No dysplasia	LSIL	HSIL+
NILM	208	167	10	19	12
ASC-US	38	25	1	4	8
LSIL	64	37	3	18	6
HSIL+	29	13	–	2	14
Total	339	242	14	43	40

ASC-US – atypical squamous cells of undetermined significance; HPV – human papillomavirus; HSIL – high-grade squamous intraepithelial lesion; LSIL – low-grade squamous intraepithelial lesion; NILM – negative for intraepithelial lesion or malignancy.

Table 4(A)
Specificity—sensitivity of reflex cytology and histology.

		Baseline histology Positive (LSIL+HSIL)	Negative
BASELINE REFLEX CYTOLOGY	**Positive**	**40**	**53**
	Negative	**43**	**203**

Sensibility: 48% Specificity: 79% Accuracy: 71%.

Table 4(B)
Colposcopic findings and histology results.

Colposcopic findings	Baseline histology No displasia	LSIL	HSIL+
Normal	–	–	–
Grade 1	10	35	7
Grade 2	0	8	33
Total	10	43	40

HSIL – high-grade squamous intraepithelial lesion; LSIL – low-grade squamous intraepithelial lesion.

Discussion

This study, based on data from an opportunistic cervical cancer screening institutional program (CHUCB) that took place between August 2012 and March 2021, showed that reflex cytology, in the presence of a positive HPV test for types 16 and 18, does not inform colposcopy practice, as most HSIL+ lesions diagnosed immediately or after 3 years of follow-up were diagnosed in women with <HSIL reflex cytology.

SurePath, the liquid medium used for transport and preservation of all samples for cytology, does not seem to introduce significant differences in terms of cutoff values when compared with other certified liquid collection media for the detection of CIN1, CIN2+, and CC lesions [13].

Three hundred thirty-nine (5.3%) of the 6376 women included in the CHUCB screening program (from August 2012 to March 2021) tested positive for HPV genotypes 16 and/or 18, which does not differ significantly from the number seen in other studies, such as ATHENA [9] and KPNC [11], although there were significant differences in the characteristics of the populations involved. Of these women, 40 (11.8%) had baseline histologic HSIL+. Only 14 (4.1%) had histologic HSIL+ over 3 years, and these results are in agreement with the studies reported in the literature. Of the 29 women with baseline HSIL+ cytology, only 14 (48.3%) had baseline histologic HSIL+.

Only women with abnormal colposcopic findings underwent biopsy, in contrast with other studies, in which a biopsy was performed in all cases. However, "blind" biopsies (4-quadrant biopsies) have not been shown to be more advantageous than colposcopy-directed biopsies, as reported by some studies [14,15].

All colposcopic examinations were performed by a single observer with accreditation by the Portuguese Colposcopy Society, so there is

always the possibility of individual bias, which may explain the low number of biopsies performed. However, there were a very small number of biopsies with no dysplasia, which suggests that there is some selectivity as to whether a biopsy is performed. It is possible that some of the HSIL lesions diagnosed in the 3 years following the first colposcopy were already present at the time and were not diagnosed, but it should be noted that in this situation, approximately 97.3% of the women had a baseline <HSIL reflex cytology.

In most of the studies published on this subject, it seems evident that there is an increased risk of histologic HSIL+ lesions for HPV-16 positive cases compared with HPV 18; in our study, this difference does not appear significant, possibly due to the small size of the sample and the characteristics of its population.

The risk of having an HSIL lesion after a positive HPV-16 and/or −18 test is greater than 4%; therefore, according to the 2019 ASCCP Guidelines [16], immediate colposcopy referral is always recommended, with no need for reflex cytology, which was not useful in informing colposcopy practice.

Conclusions

Despite the small sample and some limitations of the study, the results are nevertheless sufficient to draw some conclusions.

The assessment of the risk of HSIL lesions in the CC screening program has been fundamentally based on programs that use cotesting, with little evidence of this risk in screening programs that rely on the HPV test.

In a nationwide CC screening program, immediate colposcopy referral for all women who test positive for HPV 16 and 18 requires considerable effort. It is tempting to stratify these women into low- and high-risk groups through reflex cytology. However, this study found that reflex cytology seems not to be useful for immediate referral for colposcopy in women who test positive for HPV 16 and/or 18, as most women with histologic diagnosis of an HSIL+ lesion had <HSIL reflex cytology.

Ethics approval

The Ethics Committee of the University of Beira Interior approved the request for an opinion on this study, to which it assigned code CE-UBI-Pj-2017–027.

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CRediT authorship contribution statement

Vitor Caeiro: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Bruno Esteves:** Methodology, Validation, Supervision, Writing – review & editing. **José Fonseca-Moutinho:** Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

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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Anexo 3

Artigo: "High-Risk HPV Detection in Paraffin-Embedded Tissue from Cervical Lesions."

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High-Risk HPV Detection in Paraffin-Embedded Tissue from Cervical Lesions

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Abstract: Background: Human papillomavirus (HPV), a leading cause of cervical cancer, is present in most cases of the disease and ranks as the fourth most common cancer in women globally. Among the HPV types, fourteen (HPV 16/18/31/33/35/39/45/51/52/56/58/59/66/68) are recognized as high-risk (hrHPV), each with varying levels of oncogenic potential. Detecting and genotyping these hrHPV types in cervical lesions is crucial, requiring the development of new diagnostic methods. Methods: This study focuses on a retrospective analysis conducted on 44 women from the Cova da Beira Local Health Unit. We used the Anyplex™ II hrHPV Detection kit for hrHPV genotyping from paraffin-embedded cervical tissue samples. Results: hrHPV types were identified in 38 out of the 44 women. Genotyping revealed HPV-16 (55.3%), HPV-18/39/56/58/59 (5.3%), HPV-31 (21.1%), HPV-35 (7.9%), HPV-51/66 (2.6%), and HPV-52 (10.5%). Conclusions: This study demonstrates that the Anyplex™ II hrHPV Detection kit, originally designed for cervical cancer screening, is also effective for hrHPV genotyping in histological analyses. This methodology offers a simpler and more cost-effective approach for cervical cancer risk stratification. Its implementation in clinical practice could enhance the detection of hrHPV in cervical lesions, thereby contributing to more precise diagnoses and potentially more informed treatment strategies.

Keywords: hrHPV Detection kit; hrHPV genotyping; cervical cancer

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1. Introduction

Human papillomavirus (HPV) plays a crucial role in the development of cervical cancer, having been detected in 99.7% of all cases [1]. This high prevalence establishes HPV as the primary cause of cervical cancer and underscores the importance of targeted screening and prevention strategies. Globally, HPV is the fourth most common cause of cancer among women and is a leading cause of cancer-related mortality in 23 countries [2]. The International Agency for Research on Cancer (IARC) recognizes several HPV types as high-risk (hrHPV) due to their strong link with cervical cancer, including HPV-16/18/31/33/35/39/45/51/52/56/58/59, classified as Group 1 carcinogens. Additionally, HPV-68 and HPV-66 are categorized as probably carcinogenic (Group 2A) and possibly carcinogenic (Group 2B), respectively [3].

Screening programs employing both conventional and liquid-based cytology have contributed significantly to reducing cervical cancer incidence [4,5]. Nonetheless, hrHPV testing, particularly for hrHPV-16/18/31/33/35/39/45/51/52/56/58/59/66/68, has proven to be more sensitive than cytology in detecting high-grade intraepithelial lesions (HSILs). As a result, hrHPV testing is recommended as the primary screening tool by most scientific societies [6]. The World Health Organization (WHO) endorses a screen-and-treat approach, which includes a visual inspection with acetic acid (VIA) and hrHPV DNA tests that identify the 14 types of hrHPV [5].

In clinical practice, the risk of progressing to more severe conditions, such as cervical intraepithelial neoplasia grade 3 (CIN3+), a precursor to cervical cancer, is assessed through HPV testing and reflex cytology results. The current guidelines, including those released by the American Society for Colposcopy and Cervical Pathology (ASCCP), advocate for colposcopy when there is a 4% or greater probability of finding CIN3+ based on a combination of historical and current test results. This shift to risk-based decision making marks a significant advancement in cervical cancer screening and management, aiming to identify and treat high-grade lesions that have the potential to progress to invasive cancer [7,8].

For histological high-grade cervical lesions, validated tools to assess the risk of progression to cancer are still limited. A lower age, typically less than 25–30 years old, has often been used as a criterion to defer treatment [9]. Given that HPV types 16 and 18 are associated with more aggressive behavior and a higher risk of progression to invasive cancer [10], determining HPV types in histological lesions of HSIL could be valuable for risk stratification and treatment optimization.

In clinical practice, there is a growing need for simpler and more cost-effective techniques for HPV genotyping in histological lesions of HSIL. While hrHPV tests are commonly used to genotype samples collected from Pap tests, the identification of hrHPV genotypes in existing lesions is crucial, particularly when lesions have already progressed.

To our knowledge, this is the first time that the Anyplex™ II HPV HR Detection kit has been used for hrHPV detection in paraffin-embedded tissue from cervical lesions. This study aims to describe a protocol for DNA extraction from paraffin-embedded tissue from cervical lesions and subsequent hrHPV genotyping. This approach represents a novel application in the context of HR-HPV identification when the lesion has already occurred. This could potentially lead to improved prognosis and outcomes for patients. Additionally, our findings might support the integration of this approach into existing HPV screen-and-treat algorithms, potentially influencing future updates to HPV guidelines [8,9].

2. Results

In the present study, a total of 45 paraffin-embedded biopsy slides, each meticulously cut to a thickness of 10 µm and encapsulating HSILs, underwent genomic DNA extraction. Out of the initial 45 samples processed for genomic DNA extraction, the procedure was successfully completed in 44 samples. One sample proved to be challenging, and genomic DNA extraction was not possible. This may have been due to several possibilities like a lower amount of tissue, long-term storage, or handling error. Thus, the sample could not be considered. Therefore, the 44 successfully extracted genomic DNA samples were then genotyped for HPV using the Anyplex™ II HPV HR Detection kit, Catalog Nr. HP7E00X, (Seegene®, Seoul, Republic of Korea, acquired to Werfen, Carnaxide, Portugal).

Following the genotyping process, the collected data were analyzed using Seegene Viewer™ (Seegene® Seoul, Republic of Korea, acquired to Werfen, Carnaxide, Portugal)).

Through this analysis, it was verified that the majority of the samples, namely 38 of 44 (equating to 86.4%), tested positive for one or more hrHPV types, and 6 samples (13.6%) tested negative for hrHPV types (Table 1).

Table 1. The HPV statuses of the 44 samples included in this study.

HPV Status	*n* (%)
	44 (100)
HPV-positive	38 (86.4)
HPV-negative	6 (13.6)

In the analysis conducted in this study, the genotyping results from hrHPV detection reveal a diverse distribution of HPV types among the examined cases. In the data presented in Table 2, positivity for HPV-16 can be verified in 21 of the 38 HPV-positive samples (55.3%).

Table 2. hrHPV genotyping.

HPV Genotypes	*n* (%)
HPV-16	21 (55.3)
HPV-18	2 (5.3)
HPV-31	8 (21.1)
HPV-33	0
HPV-35	3 (7.9)
HPV-39	2 (5.3)
HPV-45	0
HPV-51	1 (2.6)
HPV-52	4 (10.5)
HPV-56	2 (5.3)
HPV-58	2 (5.3)
HPV-59	2 (5.3)
HPV-66	1 (2.6)
HPV-68	0

Furthermore, eight cases (21.1%) positive for HPV-31 were identified.

HPV-52 and HPV-35 were detected in four (10.5%) and three (7.9%) cases, respectively. The analysis also revealed lower frequencies of HPV-18, 39, 56, 58, and 59, with each of these types being found in two cases (5.3%). Lastly, the least common types detected in this cohort were HPV-51 and HPV-66, each found in only one case (2.6%).

We did not find any cases of HPV-33, HPV-45, or HPV-68 infection. This may have been due to the low estimated prevalence of these genotypes for the general female population of mainland Portugal aged 18 to 64 years (0.2%, 0.1%, and 0.2%, respectively) [11] and to the number of cases included in this study, which may have limited the number of findings.

In the present study, a significant finding was the identification of multiple hrHPV infections observed in nine cases (23.9%). The detailed data regarding multiple co-infections are clarified and summarized in Table 3.

Table 3. Co-infections of HPV genotypes.

Co-Infection	*n*
HPV-16 and HPV-18	2
HPV-16 and HPV-35	1
HPV-16 and HPV-59	1
HPV-16, HPV-31 and HPV-35	1
HPV-31 and HPV-39	2
HPV-35 and HPV-58	1
HPV-52 and HPV-56	1

Among co-infections, double hrHPV infections were the most common, identified in eight cases. Specifically, HPV-16 was involved in three distinct combinations of double infections: two cases were a co-infection with HPV-16 and HPV-18, and one case was a combination of HPV-16 with HPV-35 and of HPV-16 with HPV-59. Other double co-

infections verified were two cases positive for HPV-31 and HPV-39. Moreover, individual cases of HPV-35/58 and HPV-52/56 were found.

Remarkably, a case of triple hrHPV infection was also found, which was positive for HPV-16, HPV-31, and HPV-35.

These results indicate that the Anyplex™ II HPV HR Detection kit is feasible for genotyping cervical lesions. The use of the described protocol for DNA extraction from paraffin-embedded tissue from cervical lesions and subsequent hrHPV genotyping, which turned out, in our opinion, to be simple to execute, can be of great importance for the risk stratification of high-grade cervical lesions since the majority of the lesions were positive for hrHPV and some lesions presented multiple hrHPV infections.

3. Discussion

Cervical cancer remains a significant global health challenge, with persistent infections by high-risk human papillomavirus (hrHPV) strains, notably HPV types 16 and 18, being identified as principal risk factors. This study emphasizes the critical role of the early detection and monitoring of hrHPV infections in facilitating early interventions and potentially preventing the progression to cervical cancer.

hrHPV types are detected in the vast majority of cervical cancer cases, contributing significantly to morbidity and mortality associated with the disease [3]. Our retrospective study analyzed 45 paraffin-embedded samples from cervical lesions, focusing on the genotyping of hrHPV. For this process, DNA extraction from paraffin-embedded slides was performed, allowing for hrHPV genotyping in such tissue samples using the Anyplex™ II HPV HR Detection kit. Thus, our study underscores the precision and effectiveness of the kit in detecting hrHPV in tissue from cervical lesions.

Screening programs, especially those incorporating hrHPV testing, play a crucial role in the prevention of cervical cancer. However, our findings highlight the importance of extending hrHPV genotyping capabilities to histological analyses of cervical lesions. This approach could enhance the accuracy of cervical cancer diagnoses and aid in the stratification of patient risk, thereby informing more tailored treatment strategies.

In the present work, in 44 of 45 cases (97.8%), it was possible to extract genomic DNA for multiplex real-time PCR amplification and hrHPV detection. Among these 44 cases, 86.4% of the lesions tested positive for at least one hrHPV type. HPV-16 was the most prevalent type (55.3%), which aligns with the global epidemiological data, as a leading cause of cervical cancer, highlighting its significant oncogenic potential and the importance of its early detection [12–14].

The second most prevalent hrHPV type was HPV-31; albeit it is less commonly implicated in cervical cancer when compared to HPV-16 and HPV-18, it is also classified as a high-risk oncogenic type [3]. The prevalence of HPV-31 is in accordance with that described in the literature for Europe [15].

After that, the most common HPV types were HPV-52 and 35 (10.5% and 7.9%, respectively). The prevalence of HPV-52 was similar to that found by Sousa et al., 2019 in a population of the northern region of Portugal [16]. However, HPV-35 was present in a higher number of cases in the present cohort when compared to the study in the northern region of Portugal, and it is closer to the prevalence of HPV-35 in Africa [15,16]. The cases studied revealed lower frequencies of HPV-18, 39, 56, 58, and 59, with each of these types being found in two cases. The less frequent types were HPV-51 and HPV-66, with each being found in only one case. The prevalence of these high-risk types is similar to that identified for Europe [15].

Despite the prevalence of each high-risk type, the data indicate the need for a more accurate approach taking into account the types of HPV. The methodology presented provides information on the types of HPV that lead to precancerous lesions, going beyond a screening program that detects the prevalence of infection.

Moreover, we verified the multiple co-infections that underscore the complex interaction of the different HPV genotypes in the pathogenesis of cervical lesions and how they potentially impact the progression and management of the disease.

Almost a quarter of the lesions presented multiple hrHPV infections (23.8%). Similar results were found by Sousa et al. as 25.7% of the liquid-based cytology samples also had multiple infections [16]. hrHPV 16 was the more common type in co-infections, and there was one case of a triple co-infection (HPV-16/35/59), which illustrates the diversity of HPV interactions that can occur within the cervical epithelium, probably leading to a more heterogenic behavior in HSIL depending on the types of hrHPV present. This scenario highlights the potential for multiple high-risk HPV types to concurrently infect and influence the pathological landscape of cervical tissues. The presence of multiple hrHPV genotypes in a single case raises important questions about the interactions between different HPV types and their collective impact on the severity, progression, and treatment response of cervical lesions.

This is the first time, to our knowledge, that paraffin-embedded slides from cervical lesions were genotyped using the Anyplex™ II HPV HR Detection kit, and this represents a significant methodological advancement, offering a more streamlined and efficient approach for earlier detection of the lesions and precursors of cervical cancer development. The kit used in the present work has so far been used for biopsies of liquid cytology specimens and now shows to also be an effective tool for paraffin-embedded samples from cervical lesions.

Traditional methods for HPV detection often involve time-consuming and labor-intensive techniques. Using the referred kit for HPV detection in paraffin-embedded slides can provide a more efficient and accurate diagnostic method. This improved accuracy is vital in ensuring that individuals who are at risk receive appropriate follow-ups and treatments. The use of paraffin-embedded tissue slides is a common practice in pathology labs as they allow for the long-term storage of tissue samples.

Detecting high-risk HPV in cervical lesions has clinical relevance, giving accurate information about whether the lesion is due to hrHPV and of which type. Therefore, it can aid clinicians in the identification of patients who may require closer monitoring and more accurate treatment.

This research contributes to the ongoing efforts in cervical cancer screening and prevention. By developing a more efficient and reliable method for detecting high-risk HPV, this study may have implications for public health programs and policies aimed at reducing the burden of cervical cancer, potentially reducing the need for unnecessary interventions and minimizing healthcare costs.

Ultimately, the clinical interest of this study lies in its potential to improve patient outcomes. The early detection of high-risk HPV can lead to timely interventions, which can significantly impact the prognosis and quality of life for individuals with pre-cancerous lesions.

In summary, this study on high-risk HPV detection using the Anyplex™ II HPV HR Detection kit in paraffin-embedded slides from cervical lesions contributes to the ongoing research in cervical cancer prevention and management. While it adds information to the existing body of knowledge, the potential impact of this study on diagnostic accuracy and clinical decision making should be viewed with cautious optimism. It represents an incremental step rather than a groundbreaking advancement in the field.

The findings of this study suggest possible applications in the stratification of HSIL risk and therapeutic management. However, it is important to recognize the inherent limitations to a study of this scale. The present work is a pilot study, and further laboratory studies with a larger sample size are necessary to validate the findings and to understand the full relevance of this protocol in a clinical setting. Additionally, clinical studies are required to determine the feasibility and utility of this technique, particularly in assessing the risk progression of cervical lesions.

In essence, while this investigation provides useful insights in the context of cervical cancer screening, it highlights the utility of hrHPV genotyping HSIL biopsies, which can be performed with the developed protocol using the Anyplex™ II HPV HR Detection kit.

4. Materials and Methods

4.1. Study Population

A retrospective study was performed using paraffin-embedded tissue samples from 45 women previously submitted to squamous high-grade cervical lesion excision in Cova da Beira Local Health Unit. Sample collection occurred at the Child and Women Department, Gynaecologic Oncology Division of Cova da Beira Local Health Unit, Covilhã, Portugal. This study was approved by the Ethics Committee of Beira Interior University with the code CE-UBI-Pj-2017-027.

4.2. DNA Extraction

The meticulous process of DNA extraction from tissue samples embedded in paraffin is a critical step for molecular analysis.

Initially, prior to DNA extraction, 3 µm thick slides were stained with hematoxylin and eosin. The slides were assessed by two independent pathologists in order to confirm the presence of HSIL.

Upon confirmation of HSIL, DNA extraction was performed.

The process of preparing the slides for DNA extraction involves several meticulously executed steps to prevent cross contamination and ensure the purity of the DNA.

The first step in DNA extraction optimization was obtaining the correct thickness of the tissue; thus, 10 µm paraffin-embedded biopsy slides were obtained for each confirmed case of HSIL.

Following the xylene treatment, the slides were submerged in xylene until the slide was completely covered. Incubation was carried out using a new sterile falcon tube of 50 mL for each slide in order to avoid cross contamination. This step is crucial for deparaffinization because paraffin, which embeds and preserves tissue, must be completely dissolved, allowing for the exposure of the underlying tissue and subsequent extraction of its DNA.

The slides were then individually transferred to a new 50 mL sterile falcon tube, and absolute ethanol was used until the slide was completely submerged. The incubation period was 5 min at room temperature. After that, the slides were allowed to dry at room temperature for 5 to 10 min.

The next step involved the physical retrieval of the tissue from the slide. A new sterile scalpel was used for each sample; the tissue was carefully scraped off the slide into a 1.5 mL microtube. This is a delicate process requiring precision to ensure that the tissue is successfully collected without contamination. No flow should be present in order to avoid the escape of the scraped tissue.

The extraction of genomic DNA was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The protocol provided by the manufacturer was followed meticulously, and filtered pipette tips were used. The entire process of DNA extraction was performed in a dedicated workstation in order to maintain a contamination-free environment.

After extraction, the samples were stored at −20 °C.

4.3. HPV Genotyping

For hrHPV genotyping, the Anyplex™ II HPV HR Detection kit, Catalog Nr. HP7E00X, (Seegene®, Seoul, Republic of Korea, acquired to Werfen, Portugal) was used following the manufacturer's instructions [17]. A multiplex real-time PCR (CFX96 PCR from Bio-Rad, Hercules, CA, USA) was performed. The kit enables the simultaneous genotyping of the 14 HPV types, including 12 high-risk types identified as Group 1 carcinogens (HPV-16/18/31/33/35/39/45/51/52/56/58/59). Additionally, it encompasses HPV-66,

classified under Group 2B as possibly carcinogenic, and HPV-68, classified under Group 2A as probably carcinogenic [3,17]. The Anyplex™ II HPV HR Detection kit provides an internal control for each sample and positive and negative controls for each plate for the real-time PCR reaction. The meticulous control system is pivotal for ensuring the accuracy and reliability of the PCR results, offering an added layer of validation to the genotyping process. By including these controls, the kit effectively minimizes the potential for false positive or false negative results, thus providing a higher degree of confidence.

To prevent cross contamination, the PCR mixture was prepared within a UV PCR cabinet. Following this step, the addition of DNA was carried out in a vertical laminar flow cabinet. This cabinet ensures a sterile airflow, safeguarding the integrity of the samples and the accuracy of the genotyping process.

For genotyping, filtered pipette tips were also used, also as dedicated pipettes, to avoid cross contamination.

PCR data were analyzed using Seegene Viewer™ (Seegene®) software, Version 3, which is specifically designed to interpret data generated by multiplex real-time PCR. The internal controls of the kit and the algorithm of the software allowed us to confirm the DNA amplification and the hrHPV type(s) present in each HSIL.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

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Anexo 4

(submetido para publicação)

Artigo: "Patterns in Invasive Cervical Cancer Incidence: Exploring Factors Contributing to Higher Rates (2025)"

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Title: Patterns in Invasive Cervical Cancer Incidence: Exploring Factors Contributing to Higher Rates

Abstract

Background: Invasive cervical cancer (ICC) remains a major public health challenge with persistent disparities despite advancements in screening programs and Human Papillomavirus (HPV) vaccination. Spatial epidemiology offers a valuable tool to identify high-incidence clusters and understand contextual factors driving these disparities. This study analysed ICC trends, identified geographic clusters, and assessed associated factors in Portugal's Central Region.

Methods: This retrospective ecological study included 511 ICC cases diagnosed between 2014 and 2022 across eight public hospitals in Portugal's Central Region. Demographic, socioeconomic, and healthcare-related data were collected from official registries. Spatial analysis identified high-incidence clusters for two periods (2014-2018 and 2019-2022). Multivariable logistic regression was used to explore factors associated with these clusters.

Results: ICC incidence remained stable at 7.3/100,000 women/year, with a slight increase in 2022. The median age of diagnosis was 55 years, with 30.9% of cases occurring in women aged ≥65, who fall outside the screening target. Advanced-stage diagnoses were prevalent, particularly among older women. The spatial analysis identified two significant ICC clusters: a larger cluster of 13 municipalities during 2014–2018 (ICC rate: 13.4/100,000, relative risk [RR] = 1.97), and a smaller, more concentrated cluster of 4 municipalities during 2019–2022 (ICC rate: 21.8/100,000; RR = 3.30). Higher population density and increased healthcare availability were associated with clusters in the initial period, while lower unemployment rates were linked to clusters in the later period. Screening rates and other variables showed no statistically significant associations with clusters.

Conclusion: This study highlights significant regional disparities in ICC incidence, with evolving high-risk clusters over time. Spatial analysis is a critical tool for identifying high-risk areas, enabling targeted interventions, equitable screening access, and optimised resource allocation to reduce disparities and improve outcomes.

Keywords

Cervical cancer, Epidemiology, Spatial analysis, Geographic clustering, Uterine cervical neoplasms/ prevention and control

1. Introduction:

Invasive cervical cancer (ICC) remains a significant global public health concern, consistently ranking among the leading causes of cancer-related morbidity and mortality in women, particularly in low- and middle-resource countries [1,2]. While cervical cancer screening (CCS) programs and HPV vaccination have significantly reduced ICC incidence and mortality in high-resource settings[3,4], regional and socioeconomic disparities persist, highlighting inequities in access to preventive measures and healthcare services[4–6].

ICC incidence displays complex spatial and demographic patterns. Globally, there are peaks among younger women (aged 15–49 years)[1,4,7], while older women often present with advanced-stage disease, contributing to poorer outcomes [8–10]. Socioeconomic factors, including education, income, and healthcare access, influence ICC distribution [8,11]

Geographic variations in ICC incidence reflect complex interactions between demographic, socioeconomic, and healthcare-related factors. Studies in high-resource countries reveal that urbanized areas often report higher ICC incidence, driven by factors such as population density, access to healthcare, and delayed diagnosis in certain subgroups [3,5,12,13] Socioeconomic variables, including income inequality and education levels, also play a critical role, with disadvantaged regions often exhibiting lower screening rates and higher late-stage diagnoses [6,14].

Spatial epidemiology has proven valuable in identifying these disparities and uncovering risk determinants. A U.S.-based study identified clusters of high ICC incidence in urban areas with mixed socioeconomic profiles, suggesting that the coexistence of privileged and deprived populations influences screening participation and timely diagnosis [12] In Europe, regional studies have linked ICC disparities to variations in healthcare infrastructure and cultural attitudes toward preventive care [15,16] However, data on geographic disparities in Portugal remain limited, particularly at a municipality level.

Portugal's Central Region implemented an organised CCS program in 1990, based on cytology [17], transitioning to primary HPV screening nationally by 2019 [18]. Despite

these advancements, ICC incidence and mortality remain concerning (9.8 and 4.1/100,000 women in 2019) [7], with notable regional disparities [19].

This study applies spatial epidemiology to analyse ICC incidence in Portugal's Central Region, aiming to 1) examine temporal trends in ICC rates since 2014, 2) identify high-incidence clusters in two periods (2014–2018 and 2019–2022), and 3) determine demographic, socioeconomic, and healthcare-related factors driving these patterns.

2. Material and methods:

2.1. Study design and setting

A retrospective ecological study was conducted in Portugal's Central Region between January 2014 and December 2022. This region spans 22,635 km^2 and serves approximately 1.6 million residents [20]. The Central Region Health Administration (CHRA) oversees eight public hospitals, including a cancer centre, all equipped with colposcopy units.

The study focused on ICC cases diagnosed in CHRA public hospitals during this period. For patients treated at multiple hospitals, only records from the tertiary hospital's - Gynaecologic Oncology department - were included. Cases of recurrent disease or treated outside these public hospitals were excluded.

The CHRA transitioned to HPV screening between March and July 2019. The study period was selected based on the availability of electronic registries since 2014, allowing for a detailed analysis of ICC trends and a comparison of cytology-based screening (2014-2018), with HPV-based screening (2019-2022).

2.2. Data sources and variables:

ICC data was collected from CRHA hospital registries, after ethics and institutional approvals. Each hospital designated a co-investigator responsible for the data collection. To ensure confidentiality, data access was restricted to researchers, and all information was pseudonymised and aggregated at the municipality level.

Clinical variables included ICC diagnosis date, patient age, residence, histological type and stage at diagnosis (early: IA, IB1, IB2 and IIA1; advanced: IB3, IIA2, III and IV).

Contextual variables were selected based on their relevance in existing literature[5,14,16] and the available data. Information was sourced from official databases, like Statistics

Portugal (INE) [20] and CRHA's Information Systems (SIARS) [21], for two periods: 2014-2018 and 2019-2022, considering 2018 and 2022 as reference years for each period. All data was aggregated at the municipality level. Table 1 details the contextual variables and their sources for each period.

Demographic variables included population density, proportion of migrant residents, and age distribution of the female population. The population density was calculated as the logarithm of inhabitants per square kilometre, and age was categorised into three groups by life cycles: under 25 years, 25-64 years and 65 or older [20].

Socioeconomic variables included household income, unemployment rate, and education level. Household income, sourced from the Ministry of Finance's Income Statistics at the local level [20], used the latest available estimates from 2021 for the second period, assuming minimal changes occurred in this indicator over one year. Due to a lack of yearly estimates for unemployment rate and education level data, 2021 census data were used for both periods. Nevertheless, the use of 2021 census data ensures reliability and comprehensive coverage, enabling standardised comparisons across municipalities.

Healthcare availability metrics included the number of doctors and nurses per 1,000 residents, health units per 100,000 residents, and screening test rates. Screening test rates were calculated as the proportion of screened women to the total eligible female population (aged 25–64).

2.3. **Statistical analysis**

ICC crude rates were calculated annually, per municipality. Data was analysed in two steps: 1) identification of high ICC incidence clusters, and 2) analysis of factors associated with the clusters.

2.3.1. High ICC rate municipalities:

High ICC incidence areas in municipalities were identified using SaTScan™ software, version 9.6 (www.satscan.org), applying a discrete Poison model with a circular spatial window [22,23]. SaTScan™ was chosen for its capability to detect spatial and spatiotemporal clusters based on the discrete Poisson model, suitable for count data.

Two time periods were analysed: 2014 – 2018 (cytology-based screening), and 2019 – 2022 (HPV-based screening). The spatial window was set to include 25% of the at-risk population. This threshold allows addressing the heterogeneous population distribution

across municipalities, allowing for the detection of more precise clusters [24]. Clusters were visualised using mapping techniques.

2.3.2. Associated factors:

The identified clusters were used to determine the factors associated with high ICC rates. The outcome was binary – a municipality in a cluster, labelled "ICC clusters", or outside the clusters, labelled "non-ICC clusters".

Descriptive statistics included medians and interquartile range (IQR). Normality was assessed using Shapiro-Wilk and Kolmogorov-Smirnov tests with comparisons conducted using the Student T-test or the Mann-Whitney U test, as appropriate.

Logistic binomial generalised linear models with a logarithm link function were fitted to estimate the odds ratios (OR) and 95% confidence intervals (CI). Crude and adjusted ORs were calculated for each dimension: demographic (population density, age groups, and migrants), socioeconomic status (income, education, and unemployment), and healthcare availability variables (doctors, health units, and screening rates), for each period (2014-2018 and 2019-2022).

Statistical analyses were performed using IBM SPSS Statistics® software, version 29.0.0.0.[25]. The significance level was set at $p<0.05$.

3. Results:

3.1. Detection of ICC and demographic characteristics

Between 2014 and 2022, 624 women were diagnosed with ICC in eight public hospitals. After excluding duplicates, recurrent cases, and non-resident women, a total of 511 ICC cases were included in the study (Figure 1).

Annual ICC cases ranged from 46 (9.0%) in 2014 to 71 (13.9%) in 2022, with an average rate across municipalities of 7.3/100,000 women per year. Rates were stable over time, with a slight increase in 2022 (Figure. 2). ICC rates varied across age groups, remaining extremely low in women aged <25 years. Women aged 25-64 had the highest ICC rates across all years, ranging between 6.2 and 10.9 per 100,000 population, peaking in 2022. Among women aged ≥65 years, rates showed moderate variability, peaking in 2017, 2018 and 2022, reaching 8.7 per 100,000 (Figure 3).

The population's demographics are summarised in Table 2. The median age of diagnosis was 55 years, with 30.9% of cases in women aged ≥65 years (Figure 3). Squamous cell carcinoma (SCC) accounted for 86.9% of cases, adenocarcinoma (ADC) 12.3%, and

other histologies 0.8%. Nearly half of the cases (47.2%) were diagnosed at advanced stages, while 28.2% were at initial stages. Stage data was missing for 24.7% of cases.

3.2. High ICC rate municipalities

Spatial analysis identified two significant clusters of high ICC rates, located in coastal regions, near major urban centres (Aveiro and Viseu) [Supplementary Table 1, Figure 5.1a) and b)]. In the first period (2014-2018) a cluster covering 13 municipalities (Oliveira-de-Frades, Vouzela, São-Pedro-do-Sul, Águeda, Tondela, Albergaria-a-Velha, Viseu, Estarreja, Mortágua, Anadia, Aveiro, Oliveira-do-Bairro, and Castro-Daire) was detected. This cluster had an annual ICC incidence of 13.4 cases per 100,000 population, nearly double the expected rate (relative risk [RR] = 1.97, $p < 0.001$).

In the late period, a more focused cluster emerged, encompassing four municipalities (Águeda, Albergaria-a-Velha, Oliveira-do-Bairro, and Anadia). This cluster exhibited an annual ICC incidence of 21.8 cases per 100,000 population, with an RR of 3.3 ($p < 0.001$).

3.3. Associated Factors

Table 3 compares contextual variables across 2014-2018 and 2019-2022. The later period showed increases in women aged ≥65 (33.4% *vs* 31.7%), migrant residents (2.3% vs 1.6%), median household income (15,800 vs 13,200), and healthcare workers per 1,000 residents (doctors: 4,0 vs. 2.3; nurses: 3.4 vs. 2.1). Screening rates decreased slightly (39.9% vs 38.8%) while ICC rates and advanced disease cases were higher (13.1 and 2.4/100,000 vs 12.1 and 1.1/100,000).

We identified factors associated with municipalities classified as ICC clusters during 2014-2018 and 2019-2022 (Table 4). In the first period (2014–2018), higher population density was associated with ICC clusters (OR = 6.68; 95% CI: 1.36–32.84), but this association was not statistically significant after adjustment (adjusted OR [aOR] = 1.0; 95% CI: 0.99–1.01). Although the lower limit of the confidence interval was close to 1.00, the wide interval reflects a high degree of uncertainty, limiting definitive conclusions. A greater proportion of residents aged ≥65 years was associated with lower odds of being in an ICC cluster in the unadjusted analysis (OR = 0.87; 95% CI: 0.78–0.98). However, the adjusted analysis indicates imprecise estimates (aOR = 0.84; 95% CI: 0.71–1.00). A higher number of functional health units was linked to ICC clusters (aOR = 1.02 95% CI: 1.00–1.04), suggesting enhanced detection.

In the second period (2019–2022), population density remained associated with ICC clusters in the unadjusted analysis, but wide CIs indicates imprecision (OR = 20.30; 95% CI: 1.14–361.84; aOR = 5.32; 95% CI: 0.03–1048.44), emphasizing the uncertainty surrounding this association. Lower unemployment rates, however, were consistently linked to ICC clusters (aOR = 0.18; 95% CI: 0.04–0.92). Other variables, including education, income and screening rates showed no significant associations.

Wide CIs underscore the need for caution in interpreting these findings, as imprecision limits definitive conclusions.

4. Discussion:

This study provides new insights into ICC incidence, geographic clustering, and associated factors in Portugal's Central Region from 2014 to 2022. Two key findings emerged: first, ICC incidence remained stable overall but showed a notable increase in 2022, particularly among women aged 25–64 years. Second, spatial analysis identified evolving ICC clusters, initially widespread but increasingly concentrated near the urban centre of Aveiro in the later period. These findings emphasize the need for targeted public health efforts to reduce ICC incidence and address care inequities.

The ICC trends align with global patterns, where ICC incidence peaks in middle-aged women [4,26]. Notably, 30.9% of cases occurred in women aged ≥65 years, a group often excluded from screening programs. Advanced-stage diagnoses among older women range from 30% to 71% in high-resource countries, with significantly lower five-year survival rates compared to younger populations (23.2%–46.9% vs. 41.5%–76.7%) [9–11]. These results underscore the importance of revisiting screening age thresholds and exploring alternative approaches, such as self-sampling, to reach underserved older populations [8–10].

The high percentage of advanced-stage diagnoses (47.2%) reflects persistent challenges in early detection across Europe [14,15]. Improving screening participation and timely follow-up care, especially among older women, is critical. [14,15]. While this study aligns with broader European trends, localized data for Portugal remains scarce, underscoring the need for targeted studies to guide national policies.

Spatial analysis revealed significant ICC clusters, evolving from a larger cluster across 13 municipalities in 2014–2018, to a smaller, higher-risk cluster in four municipalities in 2019–2022. The shift likely reflects the introduction of HPV-based screening in 2019, which is more sensitive than cytology, facilitating earlier detection of precancerous lesions, particularly among younger women [6,27]. This trend mirrors experiences

reported in other countries during similar transitions, where HPV testing has improved detection rates, albeit with increased colposcopy referrals [28,29]

The COVID-19 pandemic may have further impacted ICC trends by disrupting healthcare services, delaying follow-ups, and exacerbating existing healthcare disparities [30]. While ICC is a long-standing disease, with a slow progression from high-grade lesions to invasive cancer[31,32], the pandemic's indirect effects likely influenced healthcare-seeking behaviour, particularly in underserved populations, warranting further investigation.

The identified contextual factors underscore the complexity of ICC clustering, and distinct dynamics between 2014–2018 and 2019–2022. Changes over time, such as the increasing proportion of women aged ≥65 years, a higher number of migrant residents, and improvements in healthcare infrastructure (e.g., more doctors and nurses per 1,000 residents), reflect evolving demographic and socioeconomic conditions. However, the slightly lower screening rates and higher ICC and advanced-stage diagnosis rates in the later period suggest challenges in translating these improvements into preventive care effectiveness.

Population density, significantly associated with ICC clusters in unadjusted analyses, illustrates the dual impact of urbanization. While higher density may facilitate HPV transmission due to increased social connectivity, urban areas often report lower ICC rates due to better access to healthcare and screening programs [3,13]. These mixed dynamics are further complicated by the coexistence of privileged and deprived populations within urban regions, where disparities in healthcare access and socioeconomic conditions persist[13]. Moreover, higher detection rates in areas with improved healthcare infrastructure may reflect enhanced diagnostic capacity rather than true increased disease prevalence [15,33].

The observed association of ICC clusters with improved healthcare infrastructure (e.g., the number of functional health units) suggests that enhanced diagnostic capabilities contributed to identifying cases in areas already at risk, rather than indicating preventive success[33]. Interestingly, areas with a higher proportion of older residents were less likely to form ICC clusters. This finding may reflect age-related barriers to healthcare access, as older women often face reduced healthcare-seeking behaviour, limited awareness of screening programs, and higher rates of advanced-stage diagnosis [8,10].

Lower unemployment rates in 2019-2022 may reflect improved socioeconomic conditions that facilitate healthcare access. However, the lack of significant associations with variables such as education, income, or screening rates raises questions. These

results could be influenced by methodological limitations, including reliance on aggregated data, that may obscure individual-level variations. Additionally, these results may reflect unique dynamics of ICC risk in Portugal, where the implementation of organized screening programs may have mitigated some socioeconomic disparities[3,14].

While higher education levels and income are often associated with better health literacy, increased screening participation, and reduced ICC risk[13,15], these associations were not observed in this study. This could indicate that Portugal's organized screening programs have successfully reduced disparities in access to preventive care. Nevertheless, the limitations of ecological analyses must be considered, as aggregate data may mask more nuanced, individual-level patterns. Future research incorporating longitudinal and individual-level data is critical to unravel these complexities.

These findings underscore the need for tailored interventions to address regional disparities in ICC prevention and detection. Expanding screening programs to include older populations is critical, particularly as our results and other studies demonstrate that women aged ≥65 years often present with advanced-stage disease[10,11]. Revisiting screening age thresholds and adopting alternative strategies, such as self-sampling, could help reach this underserved group more effectively [9,10]. Additionally, targeted outreach efforts in high-risk clusters should focus on increasing screening participation. Strategies such as culturally adapted campaigns and partnering with community organizations could address barriers related to healthcare access and awareness.

Integrating spatial analysis into public health surveillance can optimise resource allocation. For instance, healthcare planners could prioritise infrastructure investments, such as additional screening facilities, in underserved areas. Spatial data-driven approaches can guide targeted interventions, reducing ICC disparities and improving ICC outcomes.

5. Strengths and limitations:

A major strength of this study lies in its integration of spatial and epidemiological analyses over nine years, offering a comprehensive view of ICC trends and disparities. This long-term approach enhances the reliability of the findings and provides valuable insights for targeted interventions.

However, the study is not without limitations. Its ecological design precludes individual-level analysis, limiting the exploration of personal screening history, sexual behaviour, or HPV vaccination status - factors critical to understanding ICC risk. Additionally, reliance on hospital-based public healthcare data may exclude cases managed in private facilities, potentially introducing selection bias and excluding individuals with different socioeconomic profiles.

The use of contextual data, such as applying 2021 census data retroactively to earlier periods, may have masked temporal variations in socioeconomic factors like unemployment and education. Furthermore, missing staging data for nearly one-quarter of cases restricts the ability to fully analyse stage-related disparities.

Despite these limitations, the findings provide critical insights into regional disparities and provide a robust foundation for future research.

6. Conclusions

This study underscores significant regional disparities in ICC incidence and highlights evolving high-risk clusters in Portugal's Central Region. Despite organised screening programs and HPV vaccination, ICC rates remained steady highlighting gaps in reaching underserved populations and ensuring timely care.

The high prevalence of late-stage diagnoses, particularly among older women, calls for revising screening policies to include broader age groups and to implement alternative strategies like self-sampling. The recently updated national screening guidelines, which extend eligibility to women up to 69 years and incorporate self-sampling, represent a promising step toward addressing these gaps[34]. These changes align with our findings and could enhance participation rates, particularly in underserved and high-risk populations.

Targeted interventions in high-risk clusters remain essential for overcoming barriers and improving screening participation.

Spatial analysis has proven invaluable in guiding resource allocation and tailoring public health strategies. By integrating spatial data into routine surveillance, prevention efforts can be optimised, disparities reduced, and ICC outcomes improved, providing a clear roadmap for addressing regional disparities and enhancing control measures in the region.

7. Author statements

7.1. Ethical approval

Ethical approval was obtained from the Ethics Committee of the Central Region Health Administration and from the Ethics Committee of the Portuguese Institute of Oncology of Coimbra (IPOC), Francisco Gentil, Coimbra Hospital and University Centre (CHUC), Baixo-Vouga Hospital Centre (CHBV), Aveiro; Tondela-Viseu Hospital Centre (CHTV), Viseu; Figueira da Foz Hospital (HFF); Leiria Hospital Centre (CHL), Cova da Beira Hospital Centre (CHCB), Covilhã; Local Health Unit of Guarda (ULS-Guarda)

7.2. Funding

This work was done with no funding.

7.3. Authors' contributions

Rita Sousa: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing - Review & Editing, Visualisation, Project administration; José Alberto Fonseca-Moutinho: Conceptualisation, Methodology, Validation, Writing - Review & Editing, Project administration; Mónica Barros, Vítor Caeiro, Helena Nascimento, Sofia Pereira, Madalena Ponte, Teresa Rebelo, Isabel Saavedra, and Helena Solheiro: Investigation, Writing - Review & Editing; Fábio Gomes: Conceptualisation, Validation, Writing - Review & Editing; Fernanda Loureiro: Conceptualisation, Validation, Writing - Review & Editing; Ana Rita Goes, Conceptualisation, Methodology, Validation, Writing - Review & Editing, Project administration; Patrícia Soares: Conceptualisation, Methodology, Validation, Formal analysis, Writing - Review & Editing, Visualisation, Project administration

7.4. Declaration of competing interests

None

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9. Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version

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(6 tables; 6 figures)

Figures and Tables

Table 1: Description of contextual variables included in the analysis

Variable	Definition	Source	2014-2018	2019-2022
Demographic characteristics				

Population	• Number of residents by place of residence (2018; 2022) • Age groups categorised as <25, 25-64, and >=65years	INE, 2024	Resident Population Estimates based on the 2011 Census results	Resident Population Estimates based on the 2021 Census results
Population Density	• Logarithm of the number of residents per km² by municipality (2018; 2022)	INE, 2024	2018 estimates revised based on the 2021 Census Final results.	2022 postcensal population estimates revised using 2021 Census results
Migrant residents 'Rate	• Proportion of migrant population with legal status by municipality (2018; 2022)	INE 2023 (1)	Annual estimate	Annual estimate
Socio-economic factors				
Income	• Gross reported income per tax household (€) by municipality divided by 1,000	INE, 2024 (2)	Annual estimate for 2018	Annual estimate for 2021, assuming minimal changes in 2022
Unemployment Rate	• Proportion of unemployed residents by municipality (2021 Census)	INE, 2024 (3)	Census-based value for 2021 applied to both periods	Census-based value for 2021 applied to both periods
Education Level	• Proportion of residents with a completed high school diploma (2021 Census)	INE, 2024 (3)	Census-based value for 2021 applied to both periods	Census-based value for 2021 applied to both periods
Healthcare availability				
Doctors	• Number of medical doctors per 1,000 residents by municipality (2022)	INE, 2024 (4)	Annual estimate	Annual estimate
Health Units	• Number of functional health units per 100.000 residents, per municipality (2022)	*SIARS* – CRHA, 2023	Values based on SIARS 2022 data, applied to both periods	Values based on SIARS 2022 data, applied to both periods
Screened Population Rate	• Proportion of eligible women (25–64-years) screened in Primary Care Units within the CRHA. • Estimated based on female population by residence, age group and year (2014-2022)	SiiMA Rastreios - CRHA; INE, 2024 (1)	Screening data for 2014–2018 matched to the estimated eligible population	Screening data for 2019–2022 matched to the estimated eligible population

INE, Statistics Portugal (www.ine.pt); 1) Statistics Portugal, Annual estimates of resident population, 2) Statistics Portugal, Income Statistics at the local level produced by the Ministry of Finance - Tax and Customs Authority,3) Statistics Portugal, Population and Housing Census; 4) Statistics Portugal, Health personnel statistics; SIARS, Central Region Health Administration Information System

Table 2: demographic and clinical characteristics of ICC cases diagnosed between 2014 and 2022

	ICC (n=511)
Age, years (median; IQR)	55.0 (44.0-69.0)
Age group, years n (%)	
<25	2 (0.4%)
25-64	351 (68.7%)
• 25-34	▪ 36 (7%)
• 35-44	▪ 96 (18.8%)
• 45-54	▪ 116 (22.7%)
• 55-64	▪ 103 (20.2%)
>=65	158 (30.9%)
Histologic Type n (%)	
SCC(NOS)	444 (86,9%)
ADC	63 (12.3%)
other	4 (0.8%)
ICC Stage at diagnosis n (%)	
• Initial stage	144 (28.2%)
• Advanced stage	241 (47.2%)
• Missing	126 (24.7%)

SCC(NOS) squamous cellular cancer (not otherwise specified); ADC: adenocarcinoma

Table 3: Characterisation and comparison of contextual variables in the two time periods analysed: 2014-2018 and 2019-2022

	Earlier period 2014-2018	**Later period 2019-2022**	***p*-value**
Demographic characteristics			
Female population (n)	884,316	874,861	---
Age>=65 years (%) IQR	31.7 (26.1-36.6)	33.4 (27.6-38.5)	<0.001*
Log population density Median IQR	1.8 (1.5-2.0)	1.8 (1.4-2.0)	0.724*

Proportion of resident migrants (%) IQR	1.8 (1.5-2.0)	1.8 (1.4-2.0)	0.724*
Socioeconomic factors			
Gross reported household income / 1000 € IQR	13.2 (12.1-14.5)	15.8 (14.9-17.3)	<0.001*
Healthcare availability			
Doctors per 1000 inhabitants Median IQR	2.1 (1.5-3.2)	2.3 (1.7-3.4)	0.044*
Nurses per 1000 inhabitants Median IQR	3.4 (2.0-5.4)	4.0 (3.2-5.4)	0.001*
Screening test rates (%) IQR	39.9 (30.2-50.4)	38.8 (25.4-48.3)	0.086*

*Mann-Whitney U test. IQR – interquartile range

Table 4: Association of demographic, socioeconomic and healthcare availability with ICC Clusters

	ICC clusters 2014-2018		***ICC clusters 2019-2022***	
	OR ***(CI 95%) p-value***	***Adjusted OR (CI 95%) p-value***	**OR** ***(CI 95%) p-value***	***Adjusted OR (CI 95%) p-value***
Demographic				
Population density (Log)	6.68 (1.36-32.84) 0.02	1.00 (0.99-1.01) 0.86	20.30 (1.14-361.84) 0.04	5.32 (0.03-1048.44) 0.54
Age group >=65 years	0.87 (0.78-0.98) 0.02	0.84 (0.71-1.00) 0.05	0.79 (0.62-1.02) 0.07	0.88 (0.59-1.31) 0.52
Proportion of resident migrants (%)	1.00 (1.00-1.00) 0.42	0.53 (0.25-1.12) 0.10	1.00 (1.00-1.00) 0.74	0.94 (0.58-1.51) 0.79
Socioeconomic				
Gross reported household income/1000 (€)	1.19 (0.92-1.55) 0.19	1.19 (0.91-1.57) 0.20	1.22 (0.83-1.79) 0.31	0.59 (0.09-3.80) 0.58
Unemployment rate (%)	0.77 (0.49-1.19) 0.23	1.33 (0.85-2.08) 0.21	0.30 (0.10-0.93) 0.04	0.18 (0.04-0.92) 0.04

High-school education level (%)	1.01 (0.94-1.09) 0.78	1.01 (0.93-1.10) 0.88	1.07 (0.95-1.21) 0.25	1.30 (0.74-2.28) 0.36
Healthcare availability				
Doctors per 1000 inhabitants	0.97 (0.81-1.17) 0.78	0.87 (0.62-1.22) 0.43	0.95 (0.64 -1.42) 0.81	0.94 (0.59-1.51) 0.79
Functional Health Units per 100.000 inhabitants	1.02 (1.00-1.03) 0.02	1.02 (1.00-1.04) 0.02	1.00 (0.98-1.03) 0.88	1.00 (0.98-1.03) 0.82
Screening test rates	1.03 (0.99-1.07) 0.14	1.03 (0.98-1.07) 0.22	1.02 (0.6-1.09) 0.50	1.02 (0.99-1.05) 0.13

[1]Generalized linear model (GLM) with a binomial distribution and logarithm linkage function; [2]multivariate analysis of three models corresponding to the three dimensions of the contextual factors investigated

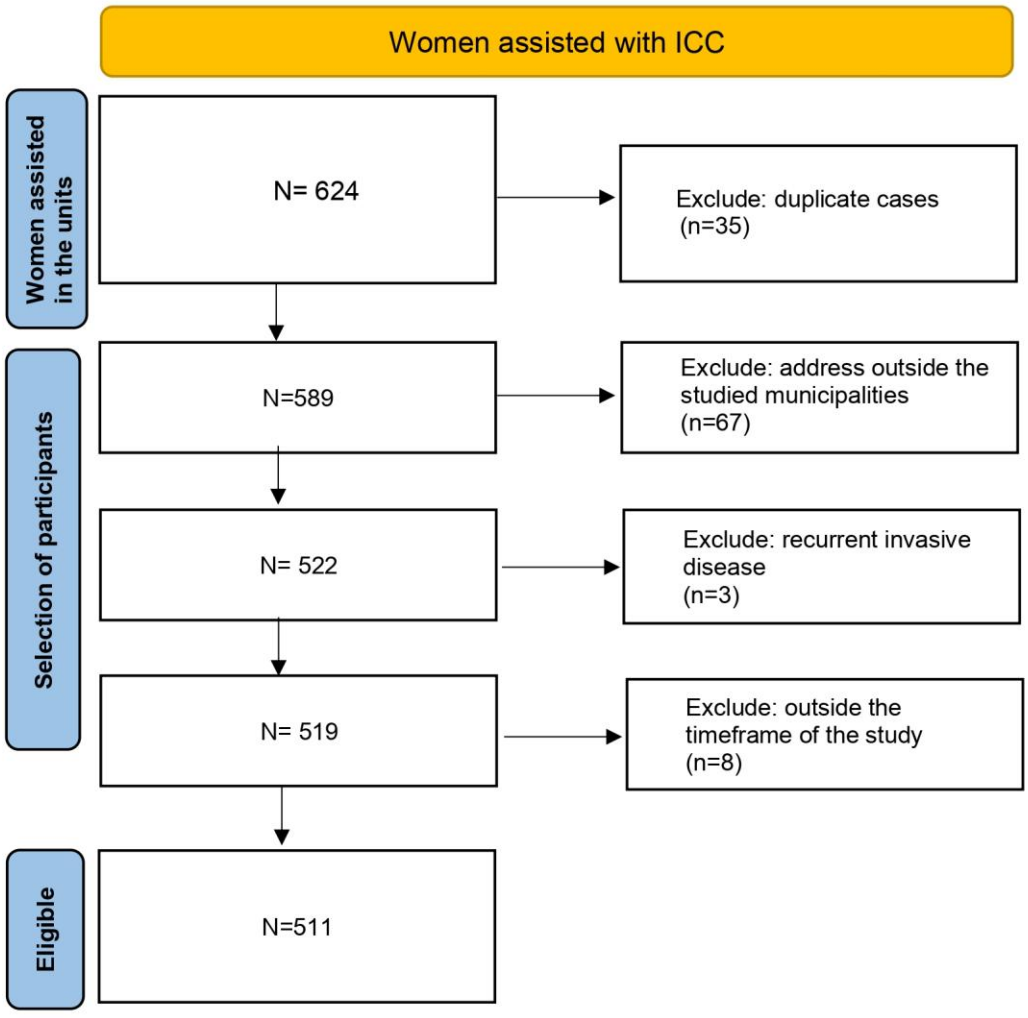


Figure 1: Flowchart of participants selection

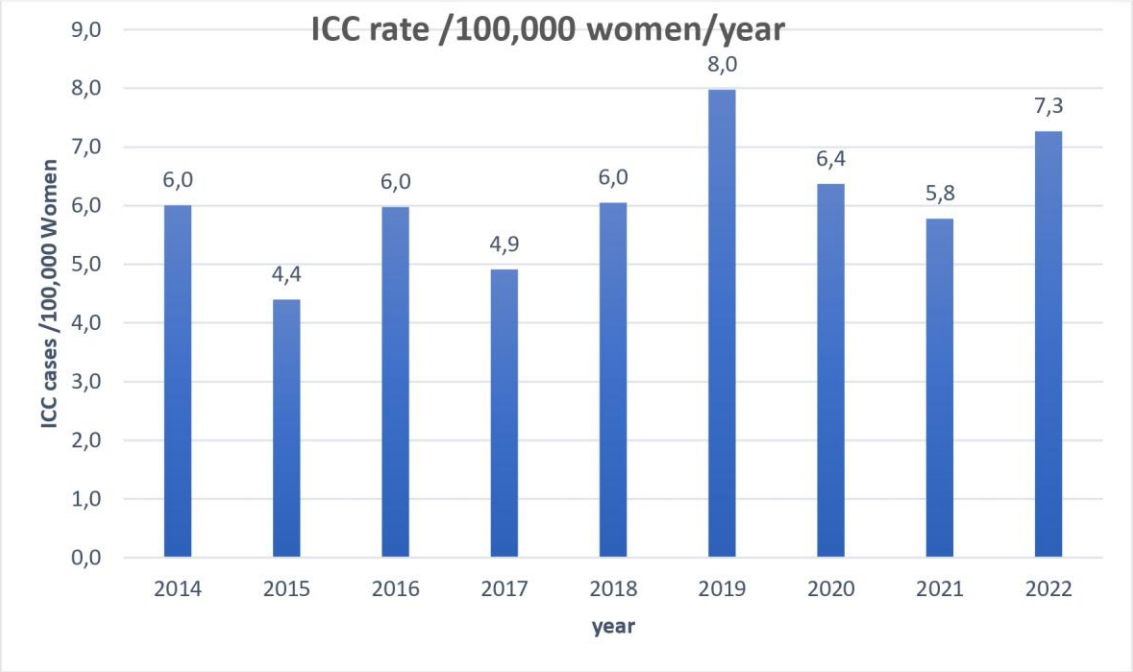
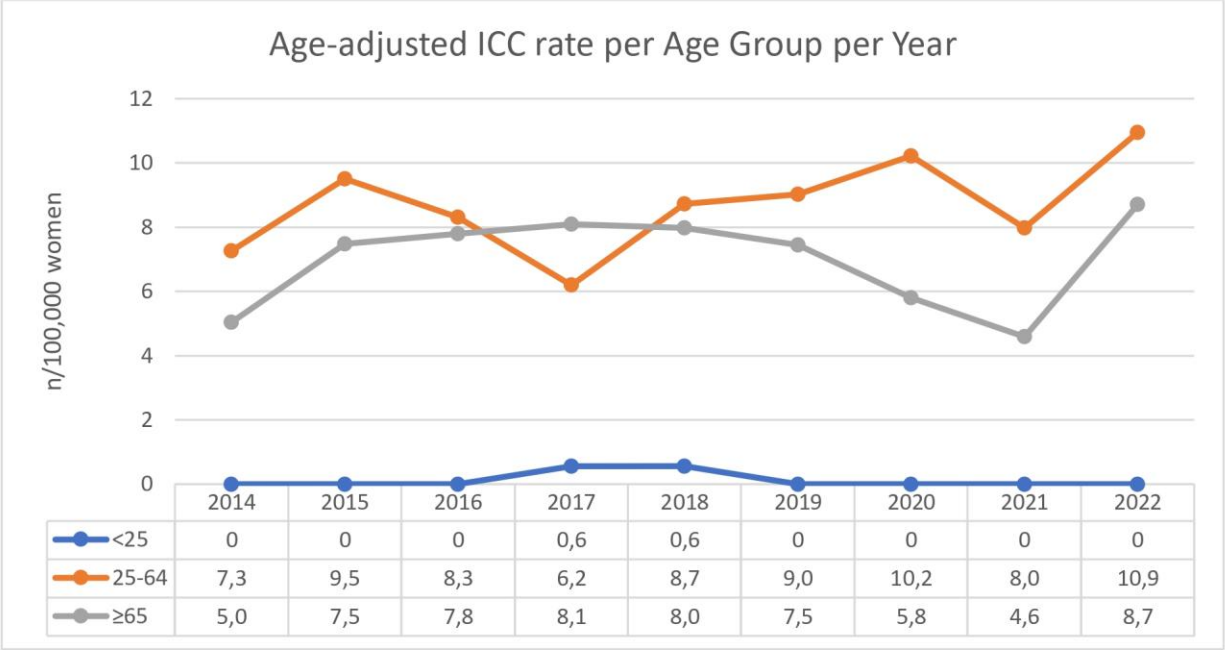


Figure 2: Annual ICC rate per 100,000 women between 2014 and 2022



	2014	2015	2016	2017	2018	2019	2020	2021	2022
<25	0	0	0	0,6	0,6	0	0	0	0
25-64	7,3	9,5	8,3	6,2	8,7	9,0	10,2	8,0	10,9
≥65	5,0	7,5	7,8	8,1	8,0	7,5	5,8	4,6	8,7

Figure 3: Annual variation of age-adjusted ICC rate /100,000 women between 2014 and 2022 by age-group (<25, 25-64 and ≥65 years)

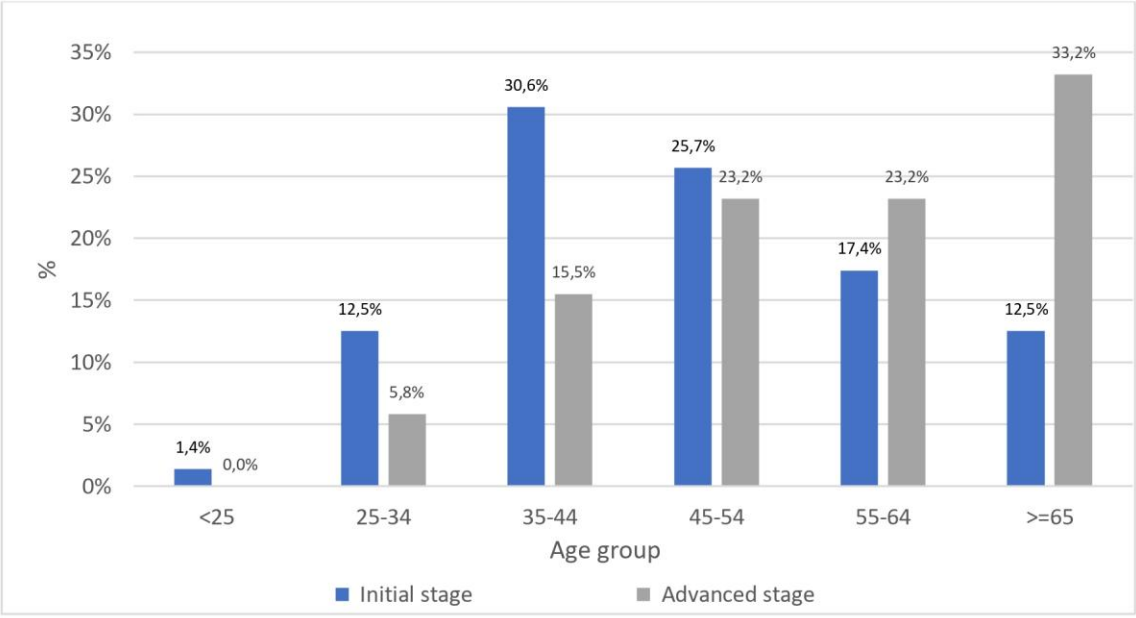


Figure 4: Stage of ICC at diagnosis between 2014 and 2022, per age group

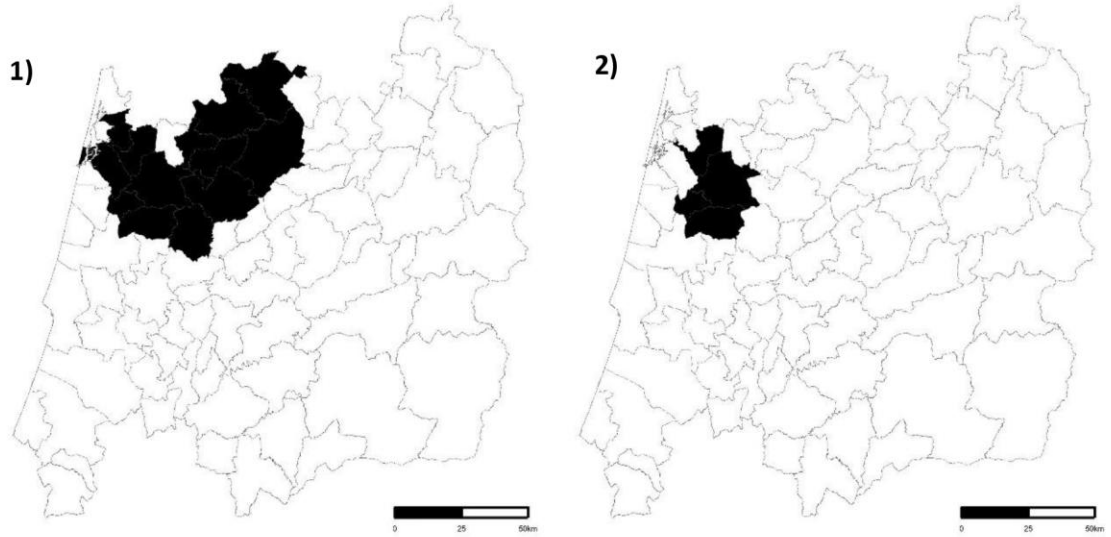


Figure 5: Spatial distribution of the areas of high ICC incidence in the Centre region of Portugal. The black dots correspond to all locations and the orange dots/circle, correspond to the clusters identified 1) early period cluster (1/1/2014-31/12/2018); 2) late period cluster (1/1/2020-31/12/2022)

Anexo 5

Parecer da Comissão de Ética

Comissão de Ética
Universidade da Beira Interior

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Convento de Santo António
6201-001 Covilhã | Portugal

Parecer relativo ao processo n.º CE-UBI-Pj-2017-027

Na sua reunião de 11 de julho de 2017 a Comissão de Ética apreciou, retrospectivamente, a documentação científica submetida referente ao pedido de parecer do projeto **"O teste de HPV como método primário de rastreio de conveniência do cancro do colo do útero"**, do proponente **Vitor Manuel Branco e Silva Caeiro**, a que atribuiu o código n.º CE-UBI-Pj-2017-027.

Na sua análise não identificou matéria que ofenda os princípios éticos e morais sendo de parecer que o estudo em causa pode ser aprovado.

Covilhã e UBI, 30 de outubro de 2017

O Presidente da Comissão de Ética

Professor Doutor José António Martinez Souto de Oliveira
Professor Catedrático