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DNA methylation biomarkers as detection and triage tools in a population-based cervical cancer screening program

Sofia Fernandes Salta

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Sofia Raquel Fernandes Salta

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE MEDICINA

Sofia Raquel Fernandes Salta

DNA METHYLATION BIOMARKERS AS DETECTION AND TRIAGE TOOLS IN A POPULATION-BASED CERVICAL CANCER SCREENING PROGRAM

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular; Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas Abel Salazar e Faculdade de Medicina)

Orientadora – **Carmen de Lurdes Fonseca Jerónimo**

Professora Catedrática Convidada do Instituto de Ciências Biomédicas Abel Salazar.
Coordenadora do Grupo de Epigenética e Biologia do Cancro & Diretora do Centro de Investigação do Instituto Português de Oncologia do Porto, Francisco Gentil E.P.E.

Co-orientador – **Rui Manuel Ferreira Henrique**

Professor Catedrático Convidado do Instituto de Ciências Biomédicas Abel Salazar.
Diretor do Serviço de Anatomia Patológica & Investigador Sénior no Grupo de Epigenética e Biologia do Cancro do Centro de Investigação do Instituto Português de Oncologia do Porto, Francisco Gentil E.P.E.

Co-orientador – **Hugo Manuel Lopes de Sousa**

Técnico Superior de Saúde no Instituto Português de Oncologia do Porto, Francisco Gentil E.P.E.

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Author Declaration

Under the terms of the “no 2, alínea a, do Art.o 31o do Decreto-lei no 230/2009”, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this doctoral thesis. Under the terms of the “no 2, alinea a, do Art.o 31o do Decreto-lei no 230/2009” is hereby declared that the following original publications were prepared within the scope of this doctoral thesis.

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*These authors contributed equally to the study.

#Joint senior authors.

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“Success is not final, failure is not fatal:
it is the courage to continue that counts”

Winston Churchill

“There's real poetry in the real world.
Science is the poetry of reality.”

Richard Dawkins

Abstract

Cervical cancer remains a public health problem worldwide. The main risk factor for cancer development is the infection by high-risk Human Papillomavirus (HrHPV). Although HrHPV infections are highly frequent, especially among young women, the majority of them are cleared by the women's immune system. However, a small fraction of women develops a persistent infection, which might progress to cancer. Primary prevention, namely through the vaccination against HPV, has an important role in the control of infections among the population. Nevertheless, secondary prevention, such as cervical cancer screening, remains crucial for the control and eradication of cervical cancer worldwide. Cervical cancer screening started based on cytology, but in the last few years, cervical cancer screening programs have been based on HrHPV detection, mainly in developed countries. Despite its high sensitivity and negative predictive value, the HrHPV test presents limited specificity, namely among young women in which transient HrHPV infections are common. This might impact the screening programs and lead to an increase in overdiagnosis and overtreatment.

In the Northern Region of Portugal, a populational-based cervical cancer screening program based on cytology was implemented in 2009. The change to the HrHPV test led to an increase in referrals to the Cervical Pathology Units, which, consequently, led to an increase in waiting time for a consultation.

This context brought an urge need to improve the triage of HrHPV-positive women. Several strategies have been proposed in this area, with DNA methylation being one of the most promising biomarkers.

In line with this problem, this Doctoral project's main aim was to develop and validate a DNA methylation-based test for the triage of HrHPV-positive women in a population-based cervical cancer screening context.

Firstly, in this Thesis, the Cervical Cancer Screening Program of Northern Portugal was characterized. The obtained results demonstrated that the cervical cancer screening program follows the high standards reported in the literature. HrHPV-based screening was shown to be more sensitive to detect high-grade squamous intraepithelial lesions or worse (HSIL+), and HPV16/18 genotyping was associated with a higher risk for these lesions. However, a portion of women sent to a colposcopy consultation did not present any lesions during the follow-up time, and some of them were able to clear the HrHPV infection.

Consequently, a systematic review and meta-analysis were conducted to disclose the potential of DNA methylation as a biomarker for the triage of HrHPV-positive women. The

results showed that DNA methylation markers might constitute an important tool to assist clinical decision-making in referring HrHPV-positive women for colposcopy either in self-collected samples or in samples collected by a health professional. *CADM1, MAL, hsa-miR124-2* and *FAM19A* were the most reported genes in the literature. Thus, these genes were selected for validation in the Portuguese population as well as in the cervical cancer screening program. Initially, the DNA methylation levels were assessed in paraffin-embedding formol-fixed samples, and three of the four genes displayed significant differences between the HSIL+ group and the control group with high specificity. These results were also observed in cervical smear samples from HrHPV-positive women, showing the great potential of DNA methylation markers in cervical cancer screening.

To validate this, a DNA methylation-based test using the droplet digital PCR, a more sensitive technique, was developed in the Doctoral project. The results of the exploratory series demonstrated that the potential of DNA methylation-based biomarkers was retained, with high specificity and accuracy for detecting HSIL+ in HrHPV-positive women. The results were validated in a consecutive series of 750 samples from the Cervical Cancer Screening Program of Northern Portugal. The biomarkers remained highly specific, and their accuracy was high. When combined with HrHPV genotyping, the sensitivity was increased.

It should also be noted that DNA methylation markers, combined with genotyping, are capable of reducing the false positives rate and the number of women sent for consultation at the Cervical Pathology Units without compromising the accuracy of the test when compared to the current strategy of triage of HrHPV positive women.

In conclusion, the work presented in this Thesis contributes to a better understanding of the current reality of the Cervical Cancer Screening Program of Northern Portugal, disclosing the main strengths and limitations and also presenting DNA methylation markers as a potential tool for the continuous improvement of the screening program, namely the triage of HrHPV-positive women.

Resumo

O cancro do colo do útero mantém-se um problema de saúde pública a nível mundial. O principal fator de risco para o desenvolvimento deste cancro é a infeção pelo Vírus do Papiloma Humano (HPV) de alto risco (HrHPV). Apesar da infeção pelo HPV ser frequente, especialmente em mulheres jovens, na sua maioria, são resolvidas pelo próprio sistema imunitário da mulher. Contudo, numa pequena percentagem de casos, a infeção persiste e pode evoluir para neoplasia. A prevenção primária, nomeadamente a vacinação contra o HPV, possui um papel importante no controlo do número destas infeções. No entanto, a prevenção secundária, como o rastreio do cancro do colo do útero, mantém um papel fundamental para o controlo e erradicação desta neoplasia. Inicialmente centrado na citologia, o rastreio de colo do útero baseia-se, atualmente, na deteção de HrHPV na maioria dos países desenvolvidos. Apesar da sua alta sensibilidade e valor preditivo negativo, o teste de HrHPV tem uma limitada especificidade, sobretudo em mulheres jovens nas quais em que as infeções transientes são frequentes, podendo conduzir a um aumento do sobre diagnóstico e sobre tratamento.

Na região Norte de Portugal, o rastreio do colo do útero de base populacional foi implementado em 2009. Esta mudança para um rastreio baseado no teste de HrHPV levou a um aumento na referenciação de mulheres para consulta de Patologia Cervical, e, consequentemente, a um aumento no tempo de espera para consulta.

Este contexto trouxe uma necessidade emergente para melhorar a triagem das mulheres positivas para HrHPV. Nos últimos anos, diversas estratégias têm vindo a ser propostas nesta área, sendo a metilação de DNA um dos biomarcadores mais promissores.

Em concordância com esta problemática, o objetivo principal deste projeto de doutoramento foi o desenvolvimento e validação de um teste de biomarcadores baseados na metilação de DNA para a triagem de mulheres HrHPV positivas no contexto do rastreio de base populacional do cancro do colo do útero.

Primeiramente, nesta tese de doutoramento foi caracterizado o programa de Rastreio do Cancro do Colo do Útero da região Norte de Portugal. Os resultados desta caraterização demonstraram que o programa de rastreio segue as melhores práticas descritas na literatura. O rastreio baseado no teste de HrHPV demonstrou ser mais sensível para a deteção de lesões escamosas intraepiteliais de alto grau ou pior (HSIL+), sendo o genótipo HPV16/18 associado a um maior risco para o desenvolvimento destas lesões. Contudo, parte das mulheres referenciadas para consulta de Patologia Cervical

não apresentaram nenhuma lesão durante o período de seguimento e, algumas delas, foram capazes de eliminar a infeção por HrHPV. Isto demonstra a necessidade de uma contínua melhoria das práticas implementadas nos programas de rastreio.

Consequentemente, foi realizada uma revisão sistemática e meta-análise dos biomarcadores de metilação de DNA com potencial para serem aplicados na triagem de mulheres HrHPV positivas. Os resultados obtidos demonstraram que os marcadores de metilação podem constituir uma importante ferramenta para auxiliar na decisão clínica de referenciar mulheres HrHPV para colposcopia, quer em amostras de auto-colheita, quer em amostras colhidas por um profissional de saúde. Os genes *CADM1, MAL, hsa-miR124-2* e *FAM19A4* foram os marcadores mais descritos na literatura. Assim, estes genes foram selecionados para validação na população portuguesa e no programa de rastreio. Inicialmente, foram avaliados os níveis de metilação de DNA em amostras fixadas em formol e incluídas em parafinas, com 3 dos 4 genes a demonstrarem diferenças entre o grupo HSIL+ e grupo controlo, com alta especificidade. Estes resultados foram também verificados em amostras cervico-vaginais de mulheres HrHPV positivas, demonstrando o potencial dos marcadores de metilação de DNA no rastreio de cancro do colo do útero.

Tendo como finalidade a validação destes biomarcadores, foi desenvolvido, neste projeto de doutoramento, um teste baseado na metilação de DNA, recorrendo a uma técnica mais sensível, o droplet digital PCR. Os resultados da série exploratória demonstraram que o potencial dos marcadores de metilação de DNA se mantinha, com alta especificidade e acuidade para a deteção de HSIL+ em mulheres HrHPV positivas. Os resultados foram validados numa série consecutiva de 750 amostras provenientes do programa de rastreio da região Norte de Portugal. Os marcadores mantiveram a sua alta especificidade e acuidade e quando conjugados com a genotipagem do HrHPV, a sensibilidade foi aumentada.

De destacar, ainda, que os marcadores de metilação de DNA associados à genotipagem são capazes de reduzir a taxa de falsos positivos e o número de mulheres enviadas a consulta em Unidade de Patologia Cervical, sem comprometer a acuidade do teste, quando comparados com a atual estratégia de triagem das mulheres HrHPV positivas.

Em conclusão, o trabalho apresentado nesta Tese contribui para conhecer melhor a realidade atual do programa de Rastreio do Cancro do Colo do Útero da região Norte de Portugal, mostrando vantagens e limitações, apresentando, ainda, marcadores de metilação de DNA como uma potencial ferramenta para a melhoria do programa de rasteio, nomeadamente na triagem de mulheres HrHPV positivas.

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List of Common Abbreviations

A

ADC – Adenocarcinoma

AGC – Atypical Glandular Cells

AIS – Adenocarcinoma in situ

ANKRD18CP – Ankyrin repeat domain 18C, pseudogene

ASC-H – Atypical Squamous Cells - cannot exclude High grade squamous intraepithelial lesion

ASCL1 – Achaete-scute family bHLH transcription factor 1

ASC-US – Atypical Squamous Cells of Undetermined Significance

ASC-US+ - ASC-US or worse

AUC – Area Under the Curve

B

Bp – base pairs

C

CADM1 – Cell adhesion molecule 1

CE-IVD – *Conformité Européene In Vitro Diagnostic*

CI – Confidence Interval

CIN – Cervical intraepithelial neoplasia

CIN2+ - CIN2 or worse

CIN3+ – CIN3 or worse

CpG – Cytosine-phosphate Guanine

D

ddMSP – Droplet digital methylation-specific PCR

ddPCR – Droplet digital PCR

DLX 1 – Distal-less homeobox 1

DNA – Deoxyribonucleic Acid

E

EPB41L3 – Erythrocyte membrane protein band 4.1 like 3

F

FAM19A4 - same as *TAFA4*

FDA – Food and Drug Administration

FFPE – Formalin-fixed paraffin-embedded

FN – False Negatives

FP – False Positives

H

HIC – High-income countries

HIV – Human Immunodeficiency Virus

HPV – Human Papillomavirus

HPV16/18+ – HPV-16 or HPV-18 positive

HrHPV – High-risk HPV

HrHPV+ - HrHPV positive

hsa-miR124-2 – microRNA 124-2

HSIL – High-grade squamous intraepithelial lesions

HSIL+ – HSIL or worse lesions

I

IARC – International Agency for Research on Cancer

ICC – Invasive cervical carcinoma

IPO Porto – Portuguese Oncology Institute of Porto

ITGA4 – Integrin subunit alpha 4

J

JAM3 – Junctional adhesion molecule 3

L

LBC – Liquid-based Cytology

LCR – Long control region

LHX8 – LIM homeobox 8

LMIC – Low-income and medium-income countries

LOB – Limit of Blank

LOD – Limit of Detection

LSIL – Low-grade squamous intraepithelial lesions

M

MAL – mal, T cell differentiation protein

miR124-2 – microRNA 124-2

mQMSP – multiplex Quantitative Methylation-Specific PCR

MSP – Methylation-specific PCR

N

NILM – Negative for Intraepithelial Lesion or Malignancy

NPV – Negative Predictive Value

P

PAX1 – Paired box 1

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

PCU – Primary Care Units

PPV – Positive Predictive Value

Q

QMSP – Quantitative Methylation-specific PCR

qPCR – Quantitative Real-Time PCR

R

RCCU – Cervical Cancer Screening Program of Northern Portugal

ROC – Receiver Operator Characteristic

RT-qPCR – Real-time quantitative PCR

RUBCNL – Rubicon-like autophagy enhancer

RXFP3 – Relaxin family peptide receptor 3

S

SCC – Squamous Cell Carcinoma

SCJ – Squamocolumnar junction

SLIT2 – Slit guidance ligand 2

SNS – National Health System

SOX 17 – SRY-box transcription factor 17

SOX1 – SRY-box transcription factor 1

SROC – Summary Receiver operating characteristics

ST6GALNAC5 – ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5

T

TAFA4 – TAFA chemokine-life family member 4

TERT – Telomerase reverse transcriptase

TN – True Negatives

TP – True Positives

TSG – Tumor Suppressor Genes

TZ – Transformation zone

U

URR – Upstream Regulatory Region

V

VIA – Visual inspection with acetic acid

W

WHO – World Health Organization

Z

ZCAN1 – Zinc finger and SCAN domain containing 1

ZNF582 – Zinc finger protein 582

ZNF671 – Zinc finger protein 671

CHAPTER I –
General Introduction

Cervical cancer

The cervix

The cervix corresponds to the lower portion of the uterus, a fibromuscular organ (Figure 1) [1]. Its length is approximately 4 cm, its diameter is about 3 cm, varying in shape and size according to women's age, hormonal state, and parity [1,2].

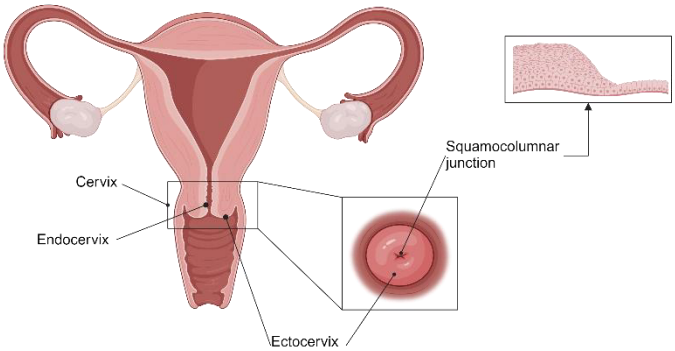

Figure 1 – Cervix anatomy. Created with BioRender.com.

A portion of the cervix projects inside the vagina and most of its surface presents a stratified squamous epithelium called ectocervix or exocervix [2]. The part of the cervix which connects the vagina to the uterine cavity is called the endocervical canal, and it is covered with a columnar epithelium named endocervix. The area where the two epitheliums are connected is designated squamous-columnar junction (SCJ) [2]. Its localization varies across women's lifetimes, which originates a new SCJ. The area between the original and the new SCJ is denominated transformation zone (TZ), corresponds to an area of metaplasia where the stratified squamous epithelium replaces the columnar epithelium with glands underlying [1,3]. TZ presents higher susceptibility for neoplastic transformation than ectocervix or endocervix [4].

Global burden of cervical cancer

Cervical cancer was reported as the 4th most frequent neoplasm among women worldwide in 2020 [5]. In 2020, 604 thousand new cases were estimated globally, with higher incidence rates in low-income and medium-income countries (LMIC), mostly localized in Africa and Asia (Figure 2) [5,6]. In high-income countries (HIC), decreased cervical cancer incidence was due to screening implementation [5,6].

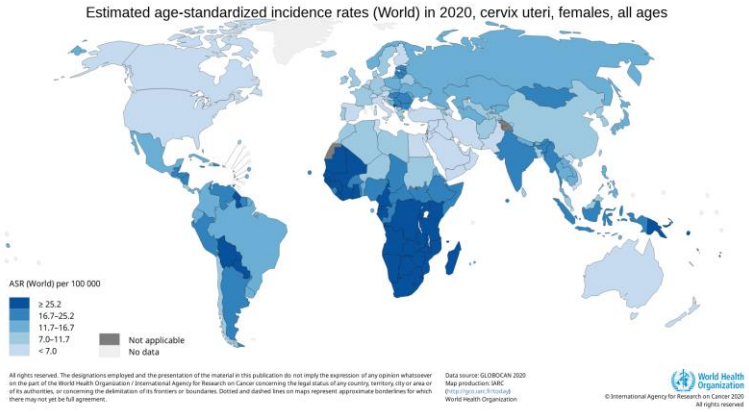

Figure 2 – Cervical cancer incidence rates worldwide in 2020. Adapted from [7].

Following the same trend, cervical cancer mortality was unequal across the globe [5,6]. In fact, it remained the deadliest cancer in 36 countries, most of them in Africa (Figure 3) [5].

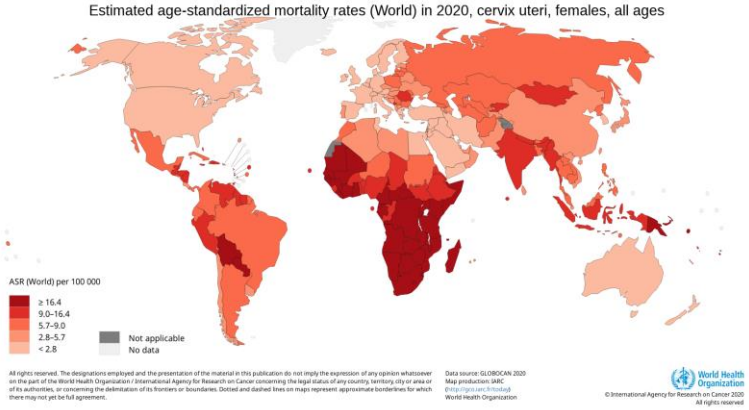

Figure 3 – Cervical cancer mortality rates worldwide in 2020. Adapted from [7].

In Portugal, the incidence rate of cervical cancer in 2019 was 9.8 per 100,000 women. Restricting only to the northern region, the rate decreases to 8.8 per 100,000 women [8]. Importantly, over the last decades, there has been a decrease in mortality rate, particularly in the decade of 1970 (Figure 4) [9].

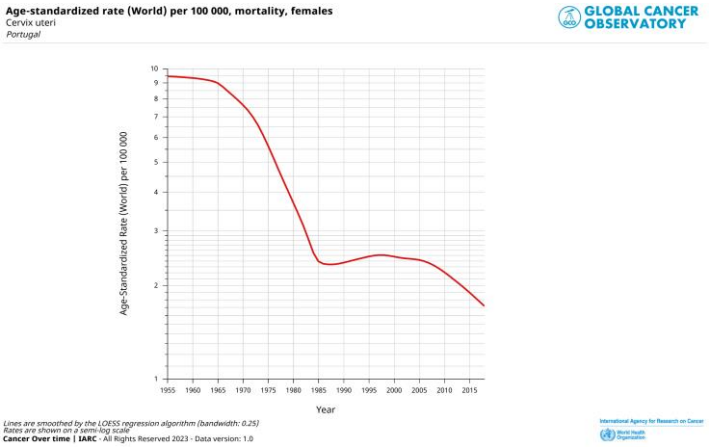

Figure 4 – Evolution of cervical cancer mortality rate in Portugal between 1955 and 2018. Adapted from [10].

This decline has been related with the increased access to heath care, including the creation of the National Health Service (SNS), enabling the performance of gynecological smear as opportunistic screening for cervical cancer since the decade of 1980 [9]. In 2019, despite the decreasing mortality rate trend in Portugal, its value was 4.1 per 100,000 women, which corresponds to a 41.8% mortality rate across cervical cancer patients [8].

Risk factors for cervical cancer

The main risk factor for cervical cancer development and its precursor lesions, classically named cervical intraepithelial neoplasia (CIN), is the infection by oncogenic Human Papillomavirus (HPV) [11]. Thus, factors that increase the probability of HPV infection might indirectly lead to a higher risk of cervical cancer development. Among these factors it is worth highlighting the high number of sexual partners, an early beginning of sexual activity and unprotected sex [12]. Although HPV infection is almost virtually needed for cancer development, *per se* is not enough. Additional risk factors are also determinant for cancer development. The International Agency for Research on Cancer (IARC) reported several co-factors that are implicated in cervical cancer development risk. Among them, exposure to tobacco smoke, high number of full-term pregnancies, and the use of oral contraceptives for more than five years are associated with a higher risk for cervical cancer development [12]. Moreover, immunosuppression and diethylstilbestrol exposure have also been reported as conditions that increase the risk of pre-cancer and cancer [11]. Additionally, other sexually transmitted infections such as *Chlamydia trachomatis* or Herpes might also increase cervical cancer risk [12].

Human Papillomavirus and neoplastic transformation

HPV genome is composed of a circular double-strand DNA with about 8,000 base pairs (bp). Its structure comprises mainly three regions: a regulatory region named long control region (LCR) (also known as Upstream Regulatory Region - URR), an early region, and a late region (Figure 5). The early region codifies six early genes (E1, E2, E4, E5, E6, and E7) related to viral replication and transcription. Otherwise, the late region codifies two genes (L1 and L2) associated with the viral capsid [12-14].

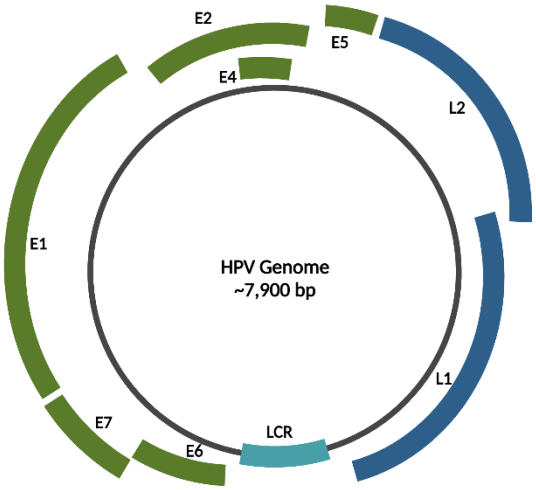

Figure 5 – Schematic of genome organization of Human Papillomavirus. Created with BioRender.

The function of these main proteins is well established (Table) and plays an essential role in viral cycle life and neoplastic transformation [12-14].

Table 1 – Function of main high-risk Human Papillomavirus. Adapted from [12-14].

Viral protein	Function
E1	Helicase activity. It is essential for viral DNA replication.
E2	Necessary for gene transcription control, namely E6 and E7, being involved in replication and genome maintenance.
E4	It interacts with the cytoskeletal protein of the host cell and might be involved in host cell disruption and viral particle release.
E5	Accessory oncoprotein, plays a supportive role in carcinogenesis.
E6	An oncoprotein that interacts with several host proteins, namely p53, leading to its degradation. It might also activate the telomerase.
E7	Oncoprotein that interacts with several host proteins, namely pRB, leading to its degradation and release of E2F protein and, consequently, cell cycle progression
L1	Major capsid protein
L2	Minor capsid protein

HPV infection is the leading risk factor for cervical cancer development, and it is estimated that it might be responsible for about 5% of all cancers [15]. HPV belongs to the *Papillomaviridae* family [16], comprising hundreds of different genotypes that can infect higher vertebrates, including humans [12]. Due to the oncogenic potential, some HPV genotypes have been classified as high-risk HPV (HrHPV), and 12 genotypes have already been classified by the IACR as carcinogenic to humans (Group 1). Concerning

cervical cancer, IARC's Group 1 encompasses HPV-16 (also considered carcinogenic in other cancer sites), HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58 and HPV-59 [17]. Additionally, HPV-68 was classified as probably carcinogenic to humans (Group 2A) [17]. Importantly, of the aforementioned genotypes, HPV-16 is considered the most oncogenic HrHPV, followed by HPV-18, which together were reported to be present in about 70% of all cervical cancers [11].

HPV infects the basal layer of the cervical epithelium. Usually, the infection occurs in the TZ of the cervix, and the viral cycle is closely related to the epithelium differentiation. Initially, the viral DNA stays unintegrated in the host genome (episomal form) with low levels of E6 and E7 highly regulated by E2 [12]. The viral replication requires the host cellular machinery, which is activated during the epithelium differentiation. During this episomal phase, also known as productive infection [18], the epithelial cells might present some cellular abnormalities associated with so-called CIN1 lesions or Low-grade Squamous Intraepithelial Lesions (LSIL) which might be resolved by the host immune system and disappear within 24 months [11,18]. Nevertheless, in about 5-10% of women infected with HrHPV, the infection fails to be eliminated by the host immune system [19].

The persistent HrHPV infection might culminate into HrHPV DNA integration in the host genome. This integration is usually associated with progression for High-grade Squamous Intraepithelial Lesion (HSIL) which includes CIN2 and CIN3 lesions and, in some cases, might culminate in cervical cancer [11,18]. Noteworthy, cervical cancer development is a multi-step process. The most common histological subtype is Squamous Cell Carcinoma (SCC), accounting for about 80% of all cervical cancers, followed by adenocarcinomas (ADC) which represent 10-20% [11,18]. Both histological subtypes are correlated with HPV integration, which is often linked to E2 disruption, which consequently enables E6 and E7 increased expression [16,20]. These two oncoproteins are essential for cancer development since they strongly contribute to inhibition of apoptosis and avoidance of cell cycle arrest through interaction with p53 and pRB (Figure 6).

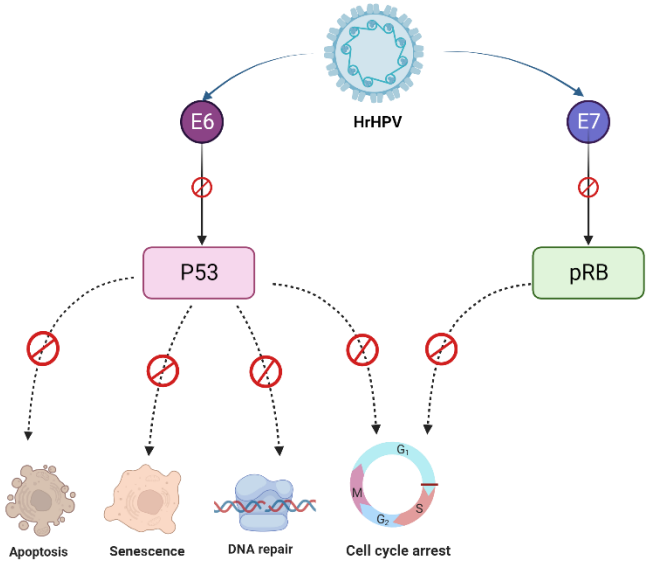

Figure 6 – Scheme of main pathways affected by Human Papillomavirus. Created with BioRender.com. Abbreviations: HrHPV – high-risk Human Papillomavirus.

E6 connects with p53 and targets it to degradation via the proteasome. Consequently, this p53 silencing leads to apoptosis and DNA repair inhibition, as well as cell cycle progression. Furthermore, E6 expression seems to trigger telomerase activity [12]. Additionally, the E7 protein has binding capacity to pRB, releasing the E2F transcription factor, allowing cell cycle progression from G1 to S-phase with DNA replication, including viral DNA replication [12,20]. The inactivation of both proteins might culminate in an aberrant and unregulated cell proliferation, which may constitute the first step for neoplastic development.

As previously mentioned, cervical cancer development is a multi-step process, so that after initial HrHPV infection, it may take 20-30 years until cancer develops, providing a "window of opportunity" for cervical cancer prevention [18].

Cervical cancer prevention

In November 2020, the World Health Organization (WHO) published a strategy to eliminate cervical cancer as a public health problem and reduce cervical cancer incidence to 4.0 per 100,000 women by the end of 2030 [21]. This global strategy is designated "90-70-90 strategy", signifying: 90% of the girls vaccinated against HPV until 15 years; 70% of women screened by 35 years of age and again at 45 years old with a

high-performance test; and 90% of women treated when diagnosed with cervical disease (with 90% precancerous lesions treated and 90% of cervical cancers managed) [21]. In line with this strategy, the European Cancer Organization published in October of the same year *"A Four Step Plan for Eliminating HPV Cancers in Europe"* [15]. This plan combines a shared effort to find effective strategies to eliminate HPV and, consequently, cervical cancer, among others, as a public health issue by 2030 [15]. Both plans (European and Global) are based on three shared fields of action: primary prevention (vaccination against HPV), secondary prevention (cancer screening), and tertiary prevention (cancer treatment and management) [15,21].

Primary Prevention – Vaccination

The main primary prevention for cervical cancer is vaccination against HPV. In 2006, Food and Drug Administration (FDA) in the United States approved the first vaccine against HPV, specifically against HPV-6, HPV-11, HPV-16, and HPV-18 [22]. Currently, five vaccine's suppliers are licensed in at least one country, summarized in Table 2.

Table 2 – Vaccines against Human Papillomavirus pre-qualified by World Health Organization. Adapted from[23,24].

Supplier	Commercial name	Genotypes covered	License approval	Schedule
Merck	Gardasil® 4-valent	HPV-6, HPV-11, HPV-16, HPV-18	Globally	2 doses (9-14 y.o.) or 3 doses (15+ y.o.)
Merck	Gardasil® 9-valent	HPV-6, HPV-11, HPV-16, HPV- 18, HPV-31, HPV-33, HPV-54, HPV-52, HPV-58	Globally	2 doses (9-14 y.o.) or 3 doses (15+ y.o.)
GSK	Cervarix®	HPV-16, HPV-18	Globally	2 doses (9-14 y.o.) or 3 doses (15+ y.o.)
SII	Cervavac®	HPV-6, HPV-11, HPV-16, HPV-18	India	2 doses (9-14 y.o.) or 3 doses (15+ y.o.)
Innovax	Cecolin®	HPV-16, HPV-18	China	2 doses (girls 9-14 y.o.) or 3 doses (girls and women 15+ y.o.)
Walvax	Walrinvax ®	HPV-16, HPV-18	China	2 doses (girls 9-14 y.o.) or 3 doses (girls and women 15-30 y.o.)

Abbreviations: HPV – Human Papillomavirus; y.o. – years old.

All the vaccines approved use virus-like particles based on DNA recombinant and cell culture instead of live agents, thus unable to cause an HPV infection [23]. The prophylactic

vaccination against HPV in young girls and women has been proven to be efficient in protecting against precancerous lesions [25]. In Portugal, all children at 10 years old are currently invited for vaccination with the 9-valent vaccine [26]. Importantly, the coverage of complete vaccination against HPV in girls born in 2005, 2006 and 2007, was above 90% [27].

Secondary Prevention – Cervical cancer screening

Despite the important role of HPV vaccination, screening is an essential pillar in the fight against cervical cancer, especially among unvaccinated women. The lifetime risk of cervical cancer development in an unvaccinated and unscreened population is about 5% [11].

In 1968, Wilson and Junger published the 10 principles for screening [28]:

1. The disease should be a major health problem.
2. The diagnosed patients must have an acceptable treatment available.
3. Infrastructures for diagnosis and treatment must be available.
4. The screened disease must have a known latent or early symptomatic stage.
5. An appropriate test or examination must exist.
6. The population must accept the test.
7. The natural history of the disease must be adequately known, including the evolution from the latent stage to a diagnosed disease.
8. An agreed policy on whom to treat should exist.
9. The screening costs (including diagnosis and treatment) must be in balance with the total economy of health care spending.
10. The screening should be continuously improved, searching for continuous refinement in screening methods and outcomes.

These principles have been the pillars of screening programs; however, not all malignancies meet all the principles, and thus, a balance between benefits and harms (such as overdiagnosis and overtreatment) is continuously searched [29]. Cervical cancer screening follows several of *Wilson & Junger's principles*. Nevertheless, cervical cancer screening has evolved over the years, based on three key points: 1) aims to select the population with a higher risk of cancer development to offer diagnosis and, if needed, treatment; 2) should be offered to the asymptomatic population, initiated by the health authorities; 3) should represent a benefit to the screened person [12].

Conventional cervical cytology was the first screening method used for cervical cancer, introduced in the 20th century after the discovery of Geórgios Papanicolau (thus known as "Pap smear") [30]. Despite its efficacy have never been assessed in randomized trials,

its introduction as screening test in a population-based program and other case-control and cohort studies proved to reduce cervical cancer incidence and mortality [30,31].

In the beginning of 21st century, several randomized trials and observational population-based studies showed that liquid-based cytology (LBC) disclosed equal or higher efficacy than conventional cytology for detection of cervical precursor lesions. LBC had a significantly lower rate of unsatisfactory results than Pap smear, simultaneously allowing for molecular tests such as HrHPV testing [30].

In the last decade and a half, HrHPV testing become the gold-standard for cervical cancer screening, demonstrating its ability to reduce cervical cancer mortality and incidence [30].

The visual inspection with acetic acid (VIA) has also been shown to reduce cervical cancer incidence and mortality in unscreened populations [30], but it is only recommended when HrHPV testing is not available, due to its limited performance [32,33].

HrHPV-based screening: recommendations, benefits and limitations

The cervical cancer screening based on HrHPV testing has shown superior performance with higher sensitivity and negative predictive value (NPV) than cytology or VIA strategies [30,32]. Due to its superiority, it is the principal strategy recommended by European Union [34] and WHO [32], leading to a change from a cytology-based program to an HrHPV-based program in most of the HIC. Notwithstanding this important change, this screening strategy must be applied only in organized population-based programs for cervical cancer screening [35,36].

An HrHPV-based screening program is recommended to begin at age 30 with screening tests each 5 to 10 years, finishing with two consecutive negative results after 50 years old [32]. The usefulness of screening test under 30 years old remains controversial with some studies reporting the benefit of starting primary HrHPV screening at 25 years old [37-39]. However, co-testing (HrHPV and cytology) is not recommended as a primary test [32,35].

Importantly, HrHPV testing as primary screening has several benefits. Those include a higher detection rate of HSIL or worse lesions (HSIL+) than the other strategies, leading to a marked decrease in cervical cancer incidence and mortality [30]. Moreover, contrarily to cytology, performance does not depend on the operator and allows for self-sampling [31].

Self-sampling screening has similar accuracy for HrHPV detection as clinician-sampling, particularly if a Polymerase Chain Reaction (PCR)-based test is used [40,41]. Moreover,

self-sampling might be key to increase cervical cancer screening uptake, especially among unscreened women [42,43].

Nevertheless, HrHPV testing has several limitations. Currently, methodologies cannot discriminate between transient and persistent HrHPV infections [11], which impacts the positive predictive value (PPV) for detecting HSIL+ [36]. Furthermore, failure to identify the type of infection negatively impacts screening programs because it increases the number of women referred to colposcopy, eventually leading to overdiagnosis and overtreatment of lesions that might spontaneously regress[11,31]. Thus, adequate triage strategies are crucial for screening programs' sustainability.

Triage strategies for HrHPV-positive women: challenges and opportunities

The high sensitivity and NPV of HrHPV testing make this strategy the gold-standard for primary cervical cancer screening. However, the inability to discriminate between transient and persistent HPV infection, associated with the high HPV infection rates, especially among young women, makes the triage strategies of HrHPV-positive women a critical issue [19]. Currently, the WHO endorses three strategies as potential triage tools for HrHPV-positive women: partial HPV genotyping (HPV 16/18), VIA and cytology [32,33]. The first two strategies are based on a screen-triage-treat approach and cytology as screen-triage for colposcopy-treat. The comparison between the three strategies, presented in Table 3, is referred to in a meta-analysis conducted by a IARC group, which only considered studies with clinician-collected samples [19].

Table 3 – Pooled sensitivity and specificity of HrHPV-positive women triage strategies to detect HSIL or worse lesions. Adapted from [19].

Triage method	No. of studies	Pooled sensitivity % (95% CI)	Pooled specificity % (95% CI)
Cytology (ASC-US+)	39	71.5 (65.2 – 77.1)	74.7 (69.2 – 79.5)
VIA	17	64.2 (56.1 – 71.5)	79.2 (73.0 – 84.2)
HPV16/18 genotyping	16	52.9 (50.2 – 55.7)	74.9 (70-3 – 79.0)
p16/Ki67 dual staining	5	80.8 (74.5 – 85.8)	69.0 (61.1 – 75.9)
HPV16/18, plus cytology (ASC-US+) in HrHPV non-HPV16/18	12	82.6 (79.2 – 85.5)	55.4 (48.2 – 62.4)
HPV16/18, plus VIA in HrHPV non-HPV16/18	2	87.2 (78.4-92.8)	59.9 (56.2 – 63.4)

Abbreviations: ASC-US+ – Atypical Squamous Cell of Undetermined Significance or worse; CI – Confidence Interval; HPV – Human Papillomavirus; HrHPV – high-risk HPV; HSIL – High-grade Squamous Intraepithelial Lesion; N – Number; VIA – Visual inspection with acetic acid.

HPV16/18 genotyping with reflex cytology in HrHPV non-HPV16/18 and p16/Ki67 dual staining are the strategies with better performance in the clinician-collected samples [19,44,45]. However, p16/Ki67 dual staining results are based on a limited number of studies and, thus, are still not endorsed by WHO as triage tool [19,32]. Moreover, significantly higher sensitivity than cytology was only verified for CIN2 or worse (CIN2+) detection, but not for CIN3 or worse (CIN3+) [19].

The second strategy, VIA, disclosed the highest specificity for HSIL+ detection. Nevertheless, high inter-study variation was observed, which might result in wide variation among programs [19]. Moreover, the use of VIA as triage for treatment results in an additional medical visit might impair screening programs feasibility.

Focusing on the third strategy, cytology is the most studied triage method. Although the pooled sensitivity and specificity above 70%, cytologists' performance is influenced by previous knowledge on HrHPV status [19,33].

Despite differences in performance among the triage strategies, cytology-based triage (including dual staining) is not directly applicable to self-sampling programs, which are increasingly under implementation to raise screening programs participation [36,46,47].

Consequently, the search for novel and more effective triage tools for HrHPV-positive women led to the identification and characterization of DNA methylation-based biomarkers [48]. Indeed, clinical trials have been carried out in The Netherlands, comparing methylation markers and cytology as triage markers in a self-sampling-based screening program [49,50]. Despite the promising results, DNA methylation-based biomarkers still lack strong evidence required by WHO to endorse its use.

The cervical cancer screening program in Northern Portugal

In Northern Portugal, a cervical cancer screening population-based program was implemented in 2010 and progressively expanded to the whole region [51,52]. All asymptomatic women, aged 25 to 60 years old, registered in primary care units (PCU) of the SNS in Northern Portugal are eligible for cervical cancer screening. The participation in this screening program is made through invitation to women in the years in which they complete 25, 30, 35, 40, 45, 50, 55, 60 and 64 years old, except if they present any of the following criteria [53]:

- Previously treated for cervical cancer;
- Previous hysterectomy;
- Did not initiated sexual activity;
- Physical inability which does not allow for gynecologic examination;

- Current symptoms or signs of gynecologic disease.

Importantly, all samples collected at the PCU are sent to a central laboratory managed by the Departments of Pathology and of Clinical Pathology at Portuguese Oncology Institute of Porto (IPO Porto). Initially, the screening program used LBC as the primary test. However, and according to European Union guidelines, the program changed to primary HrHPV-based testing on May 1st, 2017. The current flowchart of the screening program is depicted in Figure 7.

Briefly, all samples are tested for HrHPV and genotyped for 14 HPV genotypes as described [54,55]. HPV16/18-positive women are directly referred for colposcopy consultation. For positive HrHPV non-HPV16/18 samples, reflex LBC is performed. Women with Atypical Squamous Cell of Undetermined Significance (ASC-US) or worse (ASC-US+) results are also sent for colposcopy, while those testing Negative for Intraepithelial Lesion or Malignancy (NILM) are re-screened within one year. Nonetheless, change in the primary test strategy in 2017 resulted in a 4-times increase in referrals for Colposcopy Units, and, consequently, in rising waiting time for consultation and treatment [52], which undermines the screening program.

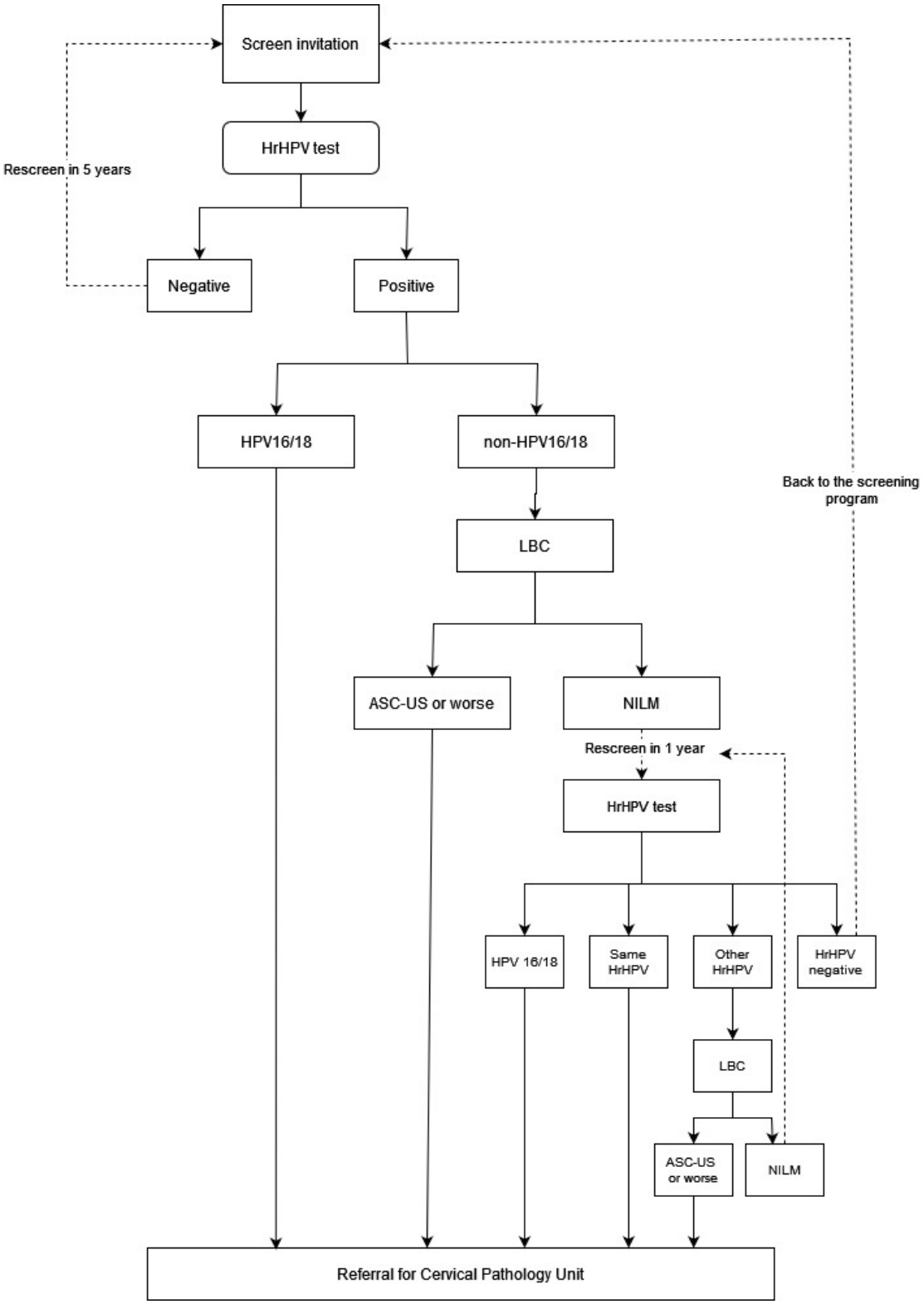

Figure 7 – Flowchart of Cervical Cancer Screening Program of Northern Portugal. Abbreviations: ASC-US: Atypical Squamous Cells of Undetermined Significance; HPV – Human Papillomavirus; HrHPV – high-risk HPV; LBC – Liquid-based Cytology; NILM – Negative for Intraepithelial Lesion or Malignancy.

DNA methylation markers in cervical cancer

DNA methylation and cancer

DNA methylation is the most studied epigenetic mechanism. Described for the first time in the late 1940s, is closely associated with gene expression regulation and genome stability. This DNA alteration in somatic cells occurs through the addition of a methyl group to the 5' position carbon of a cytosine followed by a guanine (CpG dinucleotide) [56]. These CpG dinucleotides are not randomly distributed across the genome, being predominantly localized in so-called CpGs islands. These are defined as genomic regions over 200bp, enriched for CpG content [57]. Although about 60% of gene promoters are associated with CpG islands, only some of them are usually methylated in normal cells (Figure 8). This methylation occurs during tissue development and differentiation in a tissue-specific manner [57]. Moreover, DNA methylation in CpG islands plays an important role in gene imprinting [57].

Although less expressive, DNA methylation also occurs in gene bodies, in which it is considered a mechanism to prevent inadequate transcription initiation (Figure 8) [57], as well as contributing to genome stabilization. Indeed, a significant proportion of CpG methylation is found at repetitive elements, such as transposons (Figure 8) [57].

Alterations in DNA methylation patterns have been considered emerging hallmarks of cancer [58]. During carcinogenesis, DNA methylation patterns alter. Globally, there is hypomethylation of the genome, mostly in repetitive sequences, which leads to genomic instability through endoparasitic sequence reactivation, increasing the likelihood of translocations and gene disruption (Figure 8) [57,59]. Moreover, hypomethylation at specific gene promoters might lead to inaproppriate oncogene activation and loss of imprinting. On the other hand, site-specific CpG island hypermethylation is also frequently observed, especially tumor suppressor gene (TSG) promoters (Figure 8) [57,59].

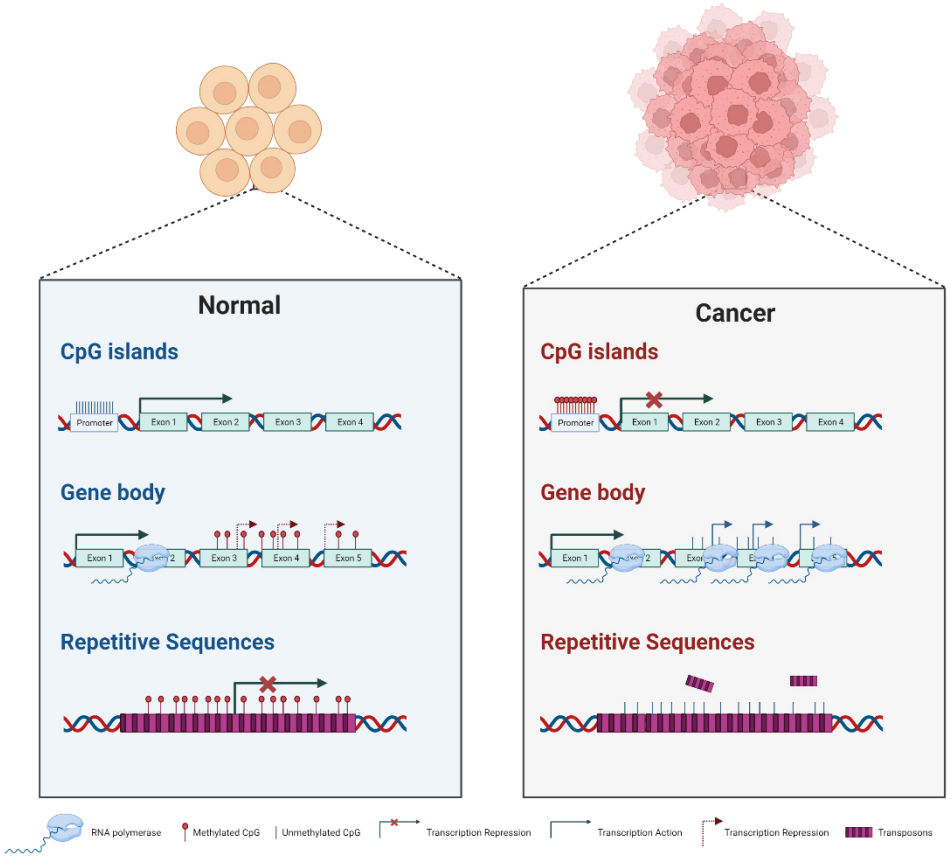

Figure 8 – DNA methylation patterns in normal and cancer cells. Created with BioRender.com.

Methylation markers in cervical cancer

Aberrant methylation patterns have been described in cervical cancer [60] and specific alterations have been proposed as biomarkers for detection, management, and monitoring. These biomarkers include both host and viral methylation sequences [60-62].

Among the most reported host genes are *cell adhesion molecule 1* (*CADM1*), *TAFA chemokine-like family member 4* (*TAFA4*, also known as *FAM19A4*), *mal, T cell differentiation protein* (*MAL*), *microRNA 124-2* (*miR124-2*), *erythrocyte membrane protein band 4.1 like 3* (*EPB41L3*), and *paired box 1* (*PAX1*) [60-62]. Although these have been the most frequently explored, HPV genome's methylation has also been reported as a potential source of biomarkers [61,62].

CADM1 is a TSG important for intracellular adhesion belonging to the immunoglobulin superfamily [59,62,63], with the methylation levels related to lesion severity [63-66]. Similar results have been reported for *MAL* [64-66], a TSG that encodes a protein involved in apical membrane transport [59,62]. Thus, *CADM1* and *MAL* methylation have been proposed as biomarkers for HrHPV-positive women [67,68]. Performance of methylation markers was reported to be non-inferior to cytology and, when combined, disclosed higher sensitivity

than cytology alone [67,68]. *CADM1* and *MAL* methylation were also reported for monitoring women treated to CIN2/3 lesions. Methylation positivity was associated with a higher risk of CIN2/3 recurrence [69].

Subsequent studies uncovered *miR124-2* methylation as additional marker, not only in cervical [59,62,70-72] but also in other cancers [59]. The 3-marker panel detected CIN3 lesions and endometrial carcinomas in cervical scrapes[71] and, in an additional study, was assessed as triage tool for HrHPV-positive women carrying Human Immunodeficiency Virus (HIV) [72]. A large Dutch clinical trial compared cytology with *MAL* and *miR124-2* methylation as a triage tool for women who tested HrHPV-positive on self-collected samples, disclosing a non-inferiority result for methylation markers [49,68].

More recently, *FAM19A4*, a gene which encodes for small proteins acting as immunoregulators [73,74], was also described as a biomarker to efficiently detect cervical cancer and advanced high-grade lesions [73]. Moreover, it was validated in large cohorts of self- and clinician-collected samples as a triage marker for HrHPV-positive women [50,74,75]. Furthermore, a panel with *miR124-2* has been widely studied. Its role as a triage marker for HrHPV-positive women and HrHPV-positive women with abnormal cytological findings has been studied in thousands of women, especially in Dutch population [76-79]. This 2-markers' methylation panel was also reported for recurrent CIN detection [80] and management of CIN3 diagnosed in pregnant women [81]. High methylation levels have been associated with higher risk of CIN3+ or CIN progression [80-82]. Remarkably, a negative result in this panel was shown to be associated with clinical regression of untreated HSIL [83].

Methylation of *PAX1*, a gene encoding a DNA binding protein, has also been reported in Asian population, especially for triage of ASC-US+ cytological findings [61].

Finally, *EPB41L3,* a TSG downregulated in cancer [84], was proposed as biomarker for detecting lesions and triaging HrHPV-positive women [61,85-87]. Moreover, S5 and S4 classifiers have also been proposed in that setting and comprise *EPB41L3* and HrHPV methylation [88,89]. The S5 classifier was reported as a potential biomarker of prognosis for CIN2 progression [90].

Although many other methylation markers have been reported across the literature as potential markers in cervical cancer detection, management, and prognosis, evidence of their performance is much less robust [60-62].

DNA methylation assessment methods

Assessment of DNA methylation status has been conducted in multiple sample types using different methodologies, which may be divided into three main categories: global

DNA methylation content, genome-wide technologies, and gene-specific technologies (Figure 9).

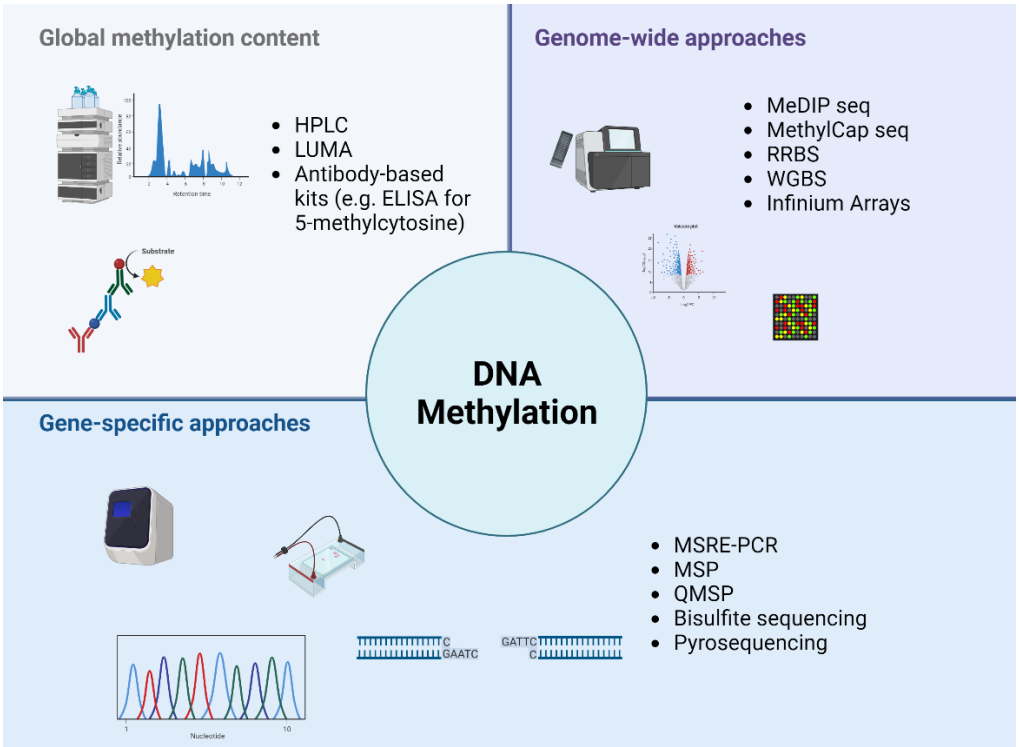

Figure 9 – Overview of the most commonly used methods for DNA methylation assessment. Created with BioRender.com. Abbreviations**:** ELISA – Enzyme-Linked Immunosorbent Assay; HPLC – High-precision liquid chromatography; LUMA – Luminometric Methylation Assay; MeDIP – Methylated DNA immunoprecipitation; MethylCap – Methylated DNA capture by affinity purification; MSP – Methylation-specific PCR; MSRE-PCR – Methylation sensitive restriction enzyme PCR; PCR – Polymerase Chain Reaction; QMSP – Quantitative Methylation-specific PCR; RRBS – Reduced representation bisulfite sequencing; seq – following by sequencing; WGBS – Whole genome bisulfite sequencing.

When a target gene is already described/known, gene-specific approaches such as Methylation-specific PCR (MSP), quantitative MSP (QMSP), bisulfite sequencing or pyrosequencing are the most used methods [91]. Gene-specific approaches usually utilize restriction enzymes or bisulfite conversion to assess the methylation status of the target sequences. Bisulfite-based methods are the most frequently used, founded on the capacity of sodium bisulfite solution to convert unmethylated cytosine into uracil (Figure 10) [92], while methylated cytosines remain as such. This allows for discrimination between methylated and unmethylated sequences using specifically designed primers and probes, targeting the methylated or the unmethylated sequences.

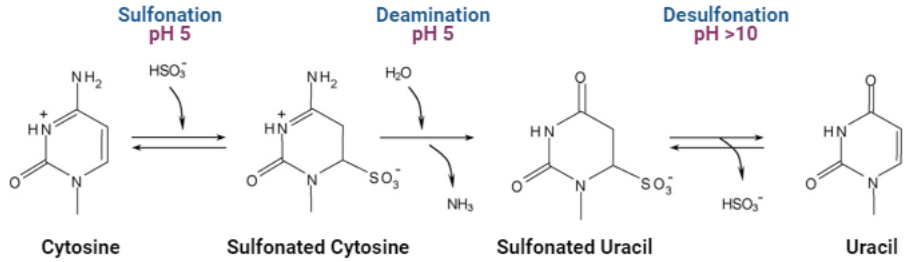

Figure 10 – Sodium Bisulfite conversion of an unmethylated cytosine to an uracil. Created with BioRender.com.

QMSP is a simple, widely used technique for assessing a target sequence's methylation status. Although it does not allow for the study of individual CpG sites, contrarily to bisulfite sequencing or pyrosequencing, it is more cost-effective and has less false-positive rates than conventional MSP [92]. It usually targets the methylated sequence with high analytical sensitivity [92] and when associated with a digital approach, the analytical sensitivity increases, especially in low-input samples [93]. Indeed, over the last year, droplet digital PCR (ddPCR) has been shown to be more accurate, reproducible and less ambiguous than Quantitative Real-Time PCR (qPCR) [94-97]. Thus, it was proposed as a more efffective alternative to QMSP in several studies [98-101].

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CHAPTER II –
Rationale and aims

Rationale and aims

The cervical cancer screening program of Northern Portugal is a population-based initiative, which started in 2009 through a pilot study and expanded for the entire region in a few years [1]. The program is fully supported by the SNS and initially used LBC as the primary test, with HrHPV testing as triage for ASC-US results, only. However, since May 2017 and according to the European guidelines, the cervical cancer screening program changed for primary HrHPV testing followed by triage with cytology in HrHPV non-HPV16/18 positive cases [2]. As a result of this change in the protocol, a fourfold increase in the number of women sent to Cervical Pathology Units was observed. This led to an overload of these units and, consequently, a heightened waiting time for consultation and treatments [2]. This not only undermines the adhesion of women (and their primary care physicians) to the screening program, but it also entails overdiagnosis and overtreatment of precursor lesions, with negative emotional and financial impact.

This doctoral project was designed with the very clear goal of investigating novel strategies to improve the efficiency of the screening program, ensuring high levels of adhesion as well as its long-term sustainability. Thus, the main aim of this thesis was to develop and validate a panel of DNA methylation-based biomarkers as a triage test for HrHPV-positive cases in the context of population-based cervical cancer screening. Specifically, the project was designed to:

1. Characterize the current screening strategy, reporting the preliminary outcomes and unveiling the areas where improvement is still required (corresponding to **Chapter III** of this Thesis).
2. Select and test the most informative methylation-based biomarkers for triage of HrHPV-positive women to referral for colposcopy (corresponding to **Chapter IV** of this Thesis).
3. Validate the most promising biomarkers in the Portuguese population (corresponding to **Chapter V** of this Thesis).
4. Innovate technologically through the adaptation and validation of a methylation-based panel for triage of HrHPV-positive women using droplet digital MSP (ddMSP) (corresponding to **Chapter VI** of this Thesis).

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CHAPTER III –
Research Paper I

Research Paper I

Paper I – Preliminary outcomes of the Cervical Cancer Screening Program of Northern Portugal: a snapshot

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Sofia Salta, José Pedro Sequeira, João Lobo, Ana Sousa, Hugo Sousa, Inês Baldaque, Paula Monteiro, Fernando Tavares, Rui Henrique[#], Carmen Jerónimo[#]

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Participation in study design, preparation and analysis of the collected data, and manuscript drafting.

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Preliminary outcomes of the Cervical Cancer Screening Program of Northern Portugal: A snapshot

Sofia Salta [a,b], José Pedro Sequeira [a,c], João Lobo [a,d,e], Ana Sousa [f,g], Hugo Sousa [f,h,i], Inês Baldaque [h], Paula Monteiro [d], Fernando Tavares [j], Rui Henrique [a,d,e,*,1,2], Carmen Jerónimo [a,e,*,1,2]

[a] Cancer Biology & Epigenetics Group, Research Center of IPO Porto (CI-IPOP), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center – Raquel Seruca (Porto.CCC) & CI-IPOP@RISE (Health Research Network), R. Dr. António Bernardino de Almeida, Porto 4200-072, Portugal
[b] Doctoral Program in Pathology and Molecular Genetics, ICBAS – School of Medicine and Biomedical Sciences – University of Porto, Rua Jorge Viterbo Ferreira 228, Porto 4050-513, Portugal
[c] Doctoral Program in Biomedical Sciences, ICBAS – School of Medicine and Biomedical Sciences – University of Porto, Rua Jorge Viterbo Ferreira 228, Porto 4050-513, Portugal
[d] Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center – Raquel Seruca (Porto.CCC) & CI-IPOP@RISE (Health Research Network), R. Dr. António Bernardino de Almeida, Porto 4200-072, Portugal
[e] Department of Pathology and Molecular Immunology, ICBAS – School of Medicine and Biomedical Sciences – University of Porto, Rua Jorge Viterbo Ferreira 228, Porto 4050–513, Portugal
[f] Molecular Oncology & Viral Pathology Group, Research Center of IPO Porto (CI-IPOP) / Porto Comprehensive Cancer Center - Raquel Seruca (Porto.CCC) & CI-IPOP@RISE (Health Research Network), R. Dr. António Bernardino de Almeida, Porto 4200-072, Portugal
[g] Escola Superior de Saúde Fernando Pessoa – ESS-FP, R. Delfim Maia 334, Porto 4200-256, Portugal
[h] Department of Pathology and Laboratory Medicine, Clinical Pathology Service, Portuguese Oncology Institute of Porto (IPO Porto), R. Dr. António Bernardino de Almeida, Porto 4200-072, Portugal
[i] Clinical Pathology Service, Centro Hospitalar Entre Douro e Vouga E.P.E., R. Dr. Cândido Pinho, Santa Maria da Feira 4520–211, Portugal
[j] Administração Regional de Saúde do Norte, I.P., Rua de Santa Catarina 1288, Porto 4000-477, Portugal

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ABSTRACT

Background: Cervical cancer screening remains an essential preventive tool worldwide. First line high-risk Human Papillomavirus (HrHPV) genotyping became gold standard for cervical cancer screening, and has been adopted by several countries, including Portugal. Herein, we aimed to assess the early outcomes of the regional Cervical Cancer Screening Program of Northern Portugal.
Methods: The analysis of a representative set of cases evaluated during a one-month period (January 2020), with adequate follow-up was performed. Descriptive analysis was performed.
Results: Overall, 7278 samples were received, of which 15.2% were HrHPV positive, most of these disclosing a negative result in subsequent liquid-based cytology. Nearly half of the HrHPV-positive women were referred to colposcopy. Within this group, HPV16/18[+] cases depicted the higher frequency of high-grade squamous intraepithelial lesion (HSIL) or worse, compared with abnormal cytology or persistent HrHPV infection. Among women with non-HPV16/18 HrHPV infection and negative cytology, which are eligible for repeat sampling in one year, 65% were re-tested. Importantly, nearly half of these cleared HrHPV infection. Furthermore, referral to colposcopy due to HPV16/18 infection and/or abnormal cytology results were associated with > 40% risk for HSIL or worse lesion.
Conclusions: Our study confirmed the reliability and effectiveness of first line HrHPV genotyping in the Cervical Cancer Screening Program of Northern Portugal. Nonetheless, it also raised concerns about

Abbreviations: ADC, Adenocarcinoma; AGC, Atypical Glandular Cells; ASC-H, Atypical Squamous Cells - cannot exclude High grade squamous intraepithelial lesion; ASC-US, Atypical squamous cells of undetermined significance; HPV, Human Papillomavirus; HrHPV, High risk Human Papillomavirus; HSIL, High-grade squamous intraepithelial lesions; HSIL+, HSIL or worse lesions; IPO Porto, Portuguese Oncology Institute of Porto; LBC, Liquid-based Cytology; LSIL, Low-grade squamous intraepithelial lesions; NILM, Negative for intraepithelial lesion or malignancy; NPV, Negative Predictive Value; PPV, Positive Predictive Value; RCCSP, Regional Cervical Cancer Screening Program of Northern Portugal; SNS, *Serviço Nacional de Saúde* – National Health Service; SCC, Squamous Cell Carcinoma; WHO, World Health Organization

* Corresponding authors at: Cancer Biology & Epigenetics Group, Research Center of IPO Porto (CI-IPOP), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center – Raquel Seruca (Porto.CCC) & CI-IPOP@RISE (Health Research Network), R. Dr. António Bernardino de Almeida, Porto 4200-072, Portugal.
E-mail addresses: henrique@ipoporto.min-saude.pt (R. Henrique), carmenjeronimo@ipoporto.min-saude.pt (C. Jerónimo).
[1] Joint Senior
[2] **Twitter:** @GEBC_IPOPorto

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excessive referral to colposcopy, with the inherent human and financial costs. Thus, further improvement and optimization are key to ensure the sustainability of the program.
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Introduction

Cervical cancer is a major health concern worldwide, accounting for 341,189 deaths among women in 2022 [1]. In Portugal, 533 new cases (167 of which in Northern Portugal) and 221 cervical cancer-related deaths (a ratio of 0.415) were recorded in 2019 [2]. High-risk Human Papillomavirus (HrHPV) infection is well established as the leading cause of cervical cancer and HPV vaccination has, thus, become the most effective primary prevention tool. Notwithstanding, cervical cancer screening remains crucial as secondary prevention, especially among unvaccinated women. Indeed, the implementation of cervical cancer screening led to a decline in incidence and mortality over the years, especially in developed countries [1,3,4].

Several European countries have implemented cervical cancer screening programs, and although only a few studies have been conducted to assess the impact of screening, a reduction in mortality was demonstrated [5]. A population-based organized cervical cancer program has been recommended to address the "Global strategy to accelerate the elimination of cervical cancer as a public health problem" proposed by the World Health Organization (WHO) with a major impact on public health [6]. This is especially important in developing countries, in which the cervical cancer burden is very high. In these populations, HrHPV testing or visual inspection with acetic acid has been shown to be more cost-effective than cytology [7]. However, some financial, cultural, and social challenges remain for the successful implementation of cervical cancer screening programs in many countries in which coverage/adherence is rather low [7].

In Northern Portugal, a regional, organized, and population-based screening program started in 2009, fully supported by the National Health Service (*Serviço Nacional de Saúde* – SNS), using cytology as a first-line test [8]. Because HrHPV testing has shown higher sensitivity and negative predictive value (NPV) [9], European guidelines [10,11] recommend HrHPV testing as first-line screening test in 2017, a strategy which was adopted by the Regional Cervical Cancer Screening Program (RCCSP) of Northern Portugal in that same year. Nonetheless, HrHPV infections are highly common, although rarely persistent, especially among young women, hampering the specificity of this screening strategy [12,13]. Consequently, the transient behavior of the virus in some women and high incidence of HrHPV infection led to an increased number of women referred to Cervical Pathology Units for colposcopic examination and, potentially, in overdiagnosis and overtreatment [12].

Currently, and since May 2017, RCCSP makes use of liquid-based cytology (LBC) for triage of non-HPV-16/18 HrHPV positive women before colposcopy referral. Although this strategy has been recommended by WHO [14] and generally acknowledged as cost-effective (even more reliable than cytology testing every 2–3 years)[15], the substantial (about 4-fold, in our experience) increase in referral to colposcopy compared to the initial strategy [first line LBC, complemented by HrHPV testing in cases diagnosed as atypical squamous cells of undetermined significance (ASC-US)] caused a significant overload of Northern Portugal Cervical Pathology Units, with consequent increase of waiting time for definitive diagnosis and treatment [16]. Thus, improving triage strategies to optimize referral of cases with high likelihood of persistent infection, and minimize overdiagnosis and overtreatment have become critical for the sustainability of the Program [17,18].

In previous publications, based on RCCSP data, we have reported on HrHPV genotype distribution and on the impact of first line HrHPV genotyping in Northern Portugal [19,20], which provided, for the first time, a real-world view on HrHPV epidemiology in the region, critically assisting in Public Health strategic decision-making. Herein, we aimed to assess the short- and mid-term clinical outcomes of women enrolled in the RCCSP, through a cross-sectional analysis of a one-month activity period, discussing potential improvements to increase cost-effectiveness and sustainability.

Material and methods

Study design

A retrospective cross-sectional study was designed based on data from RCCSP. For the specific purposes of this study, only samples received from January 1 to January 31, 2020, by the Department of Pathology of the Portuguese Oncology Institute of Porto (IPO Porto), which is the central laboratory of the whole program, in collaboration with the Clinical Pathology Service, were considered. This study is part of a larger research project which was approved by the institutional review board of IPO Porto (Comissão de Ética para a Saúde – CES-371/2017).

Study population

Data was collected from women invited by the local primary care unit to participate in the RCCSP. Cervical scrapes were collected in a methanol-based, buffered preservative solution (ThinPrep™ PreservCyt™) and sent to the Department of Pathology IPO Porto, which is the central laboratory of the whole program in collaboration with the Clinical Pathology Service. RCCSP protocol' follows the algorithm depicted in the Fig. 1.

According to RCCSP standard procedures, all samples were tested for HrHPV detection and genotyping as previously described [20]. Briefly, 14 HrHPV genotypes (HPV-16,−18,−31,−33,−35,−39,−45,−51,−52,−56,−58,−59,−66, and −68) were tested using Anyplex™ II HPV HR Detection (Seegene®, Seoul, Korea). Reflex cytology (triage) was performed, as described, in all samples that tested positive for HrHPV [19,20]. Samples were processed using ThinPrep™ 5000 Processor (HOLOGIC®, Inc.) and ThinPrep™ Imaging System (HOLOGIC®, Inc.). Dedicated cytotechnicians and cytopathologists classified all the samples according to The Bethesda System for reporting cervical cytology into one of the following categories: Negative for Intraepithelial Lesion Malignancy (NILM); ASC-US; Low-grade Squamous intraepithelial lesions (LSIL); Atypical Squamous Cells - cannot exclude High grade squamous intraepithelial lesion (ASC-H); High-grade Squamous intraepithelial lesions (HSIL); Squamous cell carcinoma (SCC); Atypical Glandular Cells (AGC) or Adenocarcinoma (ADC) [21].

Variables and data source

For all patients, HrHPV result information was collected, as well as cytological findings from HrHPV-positive samples.

For HrHPV-positive women, follow-up information was collected in the patient record, and the study was closed on December 31, 2022, completing 3 years period since the beginning of the study.

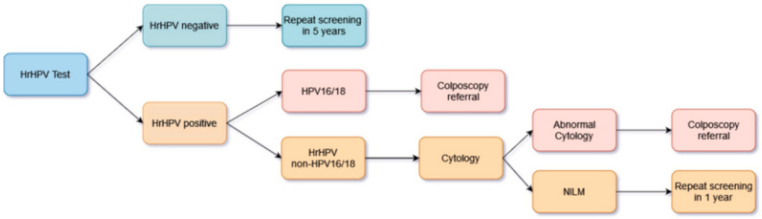

Fig. 1. – Flowchart of Regional Cervical Cancer Screening Program of Northern Portugal.

Colposcopy, HrHPV testing, cytological and histological results were retrieved whenever available, during the follow-up period.

Statistical analysis

All data was entered into a Microsoft® Excel® for Microsoft 365 MSO (version 2306 Build 16. 0. 16529. 20164) sheet. Descriptive data, tables, and figures preparation were performed with Microsoft® Excel®. Pre- and post-test probabilities were calculated as previously described [22].

Results

HPV prevalence and genotyping and cytological findings

Samples from a total of 7278 women [median age: 44 (range: 25–65) years] enrolled in the RCCSP were received by the central laboratory during the selected period. Eight samples (0.1%) had insufficient or unsatisfactory material, whereas 6166 (84.7%) tested negative for HrHPV, and the remaining 1104 (15.2%) tested positive for at least one HrHPV genotype.

HPV-31 was the most common HrHPV genotype (16.8%), followed by HPV-68 (14.6%), HPV-52 (14.1%), HPV-51 (13.8%) and HPV-16 (12.7%) (Supplementary Fig. S1). Multiple HrHPV infections were detected in 295 women (corresponding to 26.7% of all HrHPV-positive samples). As expected, younger women disclosed the highest HrHPV frequency (e.g., in women aged 25 to 29 years old, 19% of samples tested positive for HrHPV), which decreased along ageing (Supplementary Table S1, Supplementary Fig. S2).

Among HrHPV-positive women, cytological examination disclosed NILM in 791 (71.6%) cases (Supplementary Fig. S3), followed by ASC-US (n = 176, 15.9%), LSIL (n = 70, 6.3%), ASC-H (n = 37, 3.4%), and HSIL (n = 19, 1.7%). SCC was identified in only one case (0.1%) and two (0.2%) cases were classified as AGC. Eight samples (0.7%) were considered unsatisfactory for cytological examination.

Colposcopy referral and outcomes

Of the 1104 HrHPV-positive women, 540 (48.9%) were directly referred to a Cervical Pathology Unit to perform colposcopy. The main reason for referral was abnormal cytology result (ASC-US or worse, 43.1%), followed by HPV16/18 infection irrespective of cytological result (31.3%), and persistent non-HPV16/18 HrHPV infection (25.6%, which corresponded to repetitions after one year of first results). Among women referred to a Cervical Pathology Unit, 63.5% were younger than 45 years old, representing approximately 31% of all women who tested positive for HrHPV (Fig. 2).

From all women directly referred to a Cervical Pathology Unit, only 8% were lost to follow-up, and in 60% of cases a histological diagnosis was rendered based on cervical biopsy (Fig. 3). Among these, a total of 101 women disclosed HSIL or worse lesion (HSIL+), representing 19% of all women referred to colposcopy. When outcomes were analyzed according to the reason for referral, HPV16/18 was associated with higher percentage of histological result

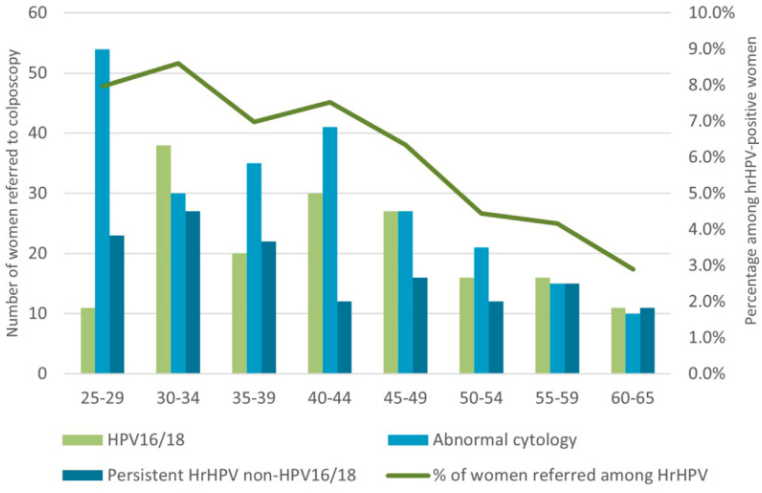

Fig. 2. – Reasons for colposcopy referral according to age group and percentage of women referred among HrHPV positive women. Abbreviations: HPV – Human Papillomavirus; HrHPV – high risk HPV.

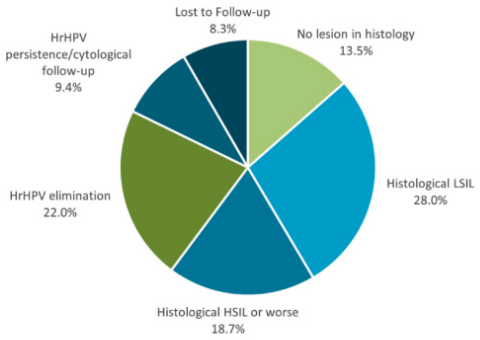

Fig. 3. – Outcomes recorded among all the women referred to a Cervical Pathology Unit. Abbreviations: HrHPV – high risk Human Papillomavirus; HSIL – High-grade Squamous intraepithelial lesions; LSIL – Low-grade Squamous intraepithelial lesions.

(representing the proportion of cases in which biopsy was performed) compared to abnormal cytology or persistent non-HPV16/18 HrHPV (72.8% vs. 57.5% vs. 49.3%, respectively) (Table 1). Moreover, in HPV16/18 + group, HSIL+ was more frequent than LSIL lesions, contrarily to that observed in the abnormal cytology or persistent non-HPV16/18 HrHPV groups [(37.3% vs. 25.4%) vs. (12.0% vs. 33.9%) vs. (7.2% vs. 21.0%), respectively]. Furthermore, HrHPV clearance was more frequent in women infected with non-HPV16/18 HrHPV than in those with abnormal cytology or HPV16/18 + groups (32.6% vs. 22.3% vs. 13.8%, respectively).

Re-screening evaluation

Of the 1104 HrHPV-positive women, 564 (51.9%) tested positive for non-HPV16/18 HrHPV and had a cytological result of NILM, of which 21% were women under 30 years (Table 2). According to the algorithm of the Program (Fig. 1), these women should repeat cervical sample collection within 1 year. A total of 365 women repeated the collection (which corresponds to 64.7% of all those eligible for repetition), 51 (9.0%) were followed-up with a different strategy (cytology or gynecology consultation), and 148 (26.2%) were lost to follow-up. Among women who repeated sample collection after one year, 155 (42.5%) had cleared HrHPV infection, 176 (48.2%) had persistent infection by the same HrHPV genotype, and 35 (9.3%) tested positive for a HrHPV genotype different from that identified in January 2020 (which was considered cleared) (Fig. 4).

At the date of study closure (December 31, 2022), among all women eligible for sample collection repetition, 205 (36.4%) cleared HrHPV within 3 years. Moreover, in this period, 112 women (20%) were biopsied and histological examination revealed HSIL+ in only 15 (2.7%) cases.

Risk-stratification

Risk stratification was calculated to determine the screening program's power. Fig. 5 and Supplementary Table S2 depict risk stratification for detection of HSIL+ using the current screening strategy (HPV 16/18 positivity and LBC positivity). Overall, a HrHPV-positive test was associated with a 10.5% probability of HSIL+. The risk increased to 37.3% if women tested positive for HPV16/18, and to 56.2% when this result was associated with an abnormal cytological result. Nonetheless, a HPV16/18 infection with NILM entailed a comparatively lower risk (24.8%). Women testing non-HPV16/18 HrHPV positive disclosed a lower risk compared to those infected with HPV16/18, regardless of cytological result (abnormal cytology: 11.9%; NILM: 3.6%).

Impact of first line HrHPV genotyping in histopathological outcome

Concerning the improvement achieved with change of primary testing in the RCCSP program, we analyzed the outcomes of NILM results (Supplementary Table S3). Of the 791 women who tested NILM in the cytology, 45 (approximately 5%) were subsequently diagnosed with HSIL+. Among NILM results, 12.1% of women tested positive for HPV16/18, 17% had a persistent HrHPV infection, and 70.1% were sent for sampling collection repetition within one year.

Table 1
– Outcomes in women directly referred to Cervical Pathology Units according to age group.

Age groups	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-65
HPV16/18 (n = 169)	n = 11	n = 38	n = 20	n = 30	n = 27	n = 16	n = 16	n = 11
Histological result [n = 123 (72.8%)]								
No lesion[a] [n = 17 (10.1%)]	1 (0.6%)	3 (1.8%)	1 (0.6%)	4 (2.4%)	3 (1.8%)	1 (0.6%)	3 (1.8%)	1 (0.6%)
LSIL [n = 43 (25.4%)]	1 (0.6%)	6 (3.6%)	4 (2.4%)	9 (5.3%)	10 (6%)	5 (3.0%)	5 (3.0%)	3 (1.8%)
HSIL or worse [n = 63 (37.3%)]	6 (3.6%)	21 (12.4%)	8 (4.7%)	10 (6%)	8 (4.7%)	5 (3.0%)	3 (1.8%)	3 (1.8%)
HrHPV clearance [n = 23 (13.6%)]	3 (1.8%)	2 (1.2%)	4 (2.4%)	2 (1.2%)	2 (1.2%)	2 (1.2%)	4 (2.4%)	4 (2.4%)
HrHPV persistence/cytological follow-up [n = 10 (5.9%)]	0 (0.0%)	2 (1.2%)	3 (1.8%)	1 (0.6%)	1 (0.6%)	2 (1.2%)	1 (0.6%)	0 (0.0%)
Lost to follow-up [n = 13 (7.7%)]	0 (0.0%)	5 (3.0%)	0 (0.0%)	4 (2.4%)	3 (1.8%)	1 (0.6%)	0 (0.0%)	0 (0.0%)
Abnormal Cytology (n = 233)	n = 54	n = 30	n = 35	n = 41	n = 27	n = 21	n = 15	n = 10
Histological result [n = 134 (57.5%)]								
No lesion[a] [n = 27 (11.6%)]	5 (2.1%)	0 (0.0%)	6 (2.6%)	7 (3.0%)	5 (2.1%)	3 (1.3%)	0 (0.0%)	1 (0.4%)
LSIL [n = 79 (33.9%)]	20 (8.6%)	6 (2.6%)	15 (6.4%)	13 (5.6%)	11 (4.7%)	3 (1.3%)	4 (1.7%)	7 (3.0%)
HSIL or worse [n = 28 (12.0%)]	6 (2.6%)	5 (2.1%)	3 (1.3%)	5 (2.1%)	2 (0.9%)	4 (1.7%)	2 (0.9%)	1 (0.4%)
HrHPV clearance [n = 52 (22.3%)]	10 (4.3%)	11 (4.7%)	6 (2.6%)	10 (4.3%)	5 (2.1%)	4 (1.7%)	6 (2.6%)	0 (0.0%)
HrHPV persistence/cytological follow-up [n = 25 (10.7%)]	6 (2.6%)	1 (0.4%)	5 (2.1%)	4 (1.7%)	3 (1.3%)	2 (0.9%)	3 (1.3%)	1 (0.4%)
Lost to follow-up [n = 22 (9.4%)]	7 (3.0%)	7 (3.0%)	0 (0.0%)	2 (0.9%)	1 (0.4%)	5 (2.1%)	0 (0.0%)	0 (0.0%)
Persistent HrHPV non-HPV16/18 (n = 138)	n = 23	n = 27	n = 22	n = 12	n = 16	n = 12	n = 15	n = 11
Histological result [n = 68 (49.3%)]								
No lesion[a] [n = 29 (21.0%)]	6 (4.3%)	3 (2.2%)	7 (5.1%)	1 (0.7%)	2 (1.4%)	3 (2.2%)	4 (2.9%)	3 (2.2%)
LSIL [n = 29 (21.0%)]	2 (1.4%)	6 (4.3%)	6 (4.3%)	2 (1.4%)	3 (2.2%)	3 (2.2%)	4 (2.9%)	3 (2.2%)
HSIL or worse [n = 10 (7.2%)]	2 (1.4%)	3 (2.2%)	1 (0.7%)	1 (0.7%)	1 (0.7%)	2 (1.4%)	0 (0.0%)	0 (0.0%)
HrHPV clearance [n = 45 (32.6%)]	6 (4.3%)	9 (6.5%)	7 (5.1%)	6 (4.3%)	7 (5.1%)	2 (1.4%)	6 (4.3%)	2 (1.4%)
HrHPV persistence/cytological follow-up [n = 15 (10.9%)]	4 (2.9%)	4 (2.9%)	1 (0.7%)	2 (1.4%)	2 (1.4%)	0 (0.0%)	1 (0.7%)	1 (0.7%)
Lost to follow-up [n = 10 (7.2%)]	3 (2.2%)	2 (1.4%)	0 (0.0%)	0 (0.0%)	1 (0.7%)	2 (1.4%)	0 (0.0%)	2 (1.4%)

[a] – In this group are included cervicitis, metaplasia and the normal histology. **Abbreviations**: HPV - Human Papillomavirus; HrHPV - high-risk HPV; HSIL - High-grade Squamous Intraepithelial Lesion; LSIL - Low-grade Squamous Intraepithelial Lesion

Table 2
– Outcomes among women which repeated sample collection within one year, according to age group.

Age Group	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-65
Total, n (%)	120 (21.3%)	84 (14.9%)	87 (15.4%)	70 (12.4%)	65 (11.5%)	57 (10.1%)	44 (7.8%)	37 (6.6%)
Histological result [n = 112 (19.9%)]								
No lesion[a] [n = 34 (6.0%)]	5 (0.9%)	7 (1.2%)	1 (0.2%)	6 (1.1%)	5 (0.9%)	5 (0.9%)	2 (0.4%)	3 (0.5%)
LSIL [n = 63 (11.2%)]	16 (2.8%)	8 (1.4%)	10 (1.8%)	9 (1.6%)	8 (1.4%)	6 (1.1%)	3 (0.5%)	3 (0.5%)
HSIL or worse [n = 15 (2.7%)]	5 (0.9%)	0 (0.0%)	2 (0.4%)	4 (0.7%)	1 (0.2%)	0 (0.0%)	2 (0.4%)	1 (0.2%)
HrHPV elimination in screening repetition [n = 171 (30.3%)]	35 (6.2%)	22 (3.9%)	30 (5.3%)	19 (3.4%)	19 (3.4%)	17 (3.0%)	13 (2.3%)	16 (2.8%)
HrHPV elimination within 3 years [n = 34 (6.0%)]	2 (0.4%)	4 (0.7%)	5 (0.9%)	6 (1.1%)	6 (1.1%)	5 (0.9%)	5 (0.9%)	1 (0.2%)
HrHPV infection/cytological follow-up [n = 99 (17.6%)]	26 (4.6%)	15 (2.7%)	19 (3.4%)	11 (2.0%)	11 (2.0%)	9 (1.6%)	4 (0.7%)	4 (0.7%)
Lost to follow-up [n = 148 (26.2%)]	31 (5.5%)	28 (5.0%)	20 (3.5%)	15 (2.7%)	15 (2.7%)	15 (2.7%)	15 (2.7%)	9 (1.6%)

[a] – In this group are included cervicitis, metaplasia and the normal histology. **Abbreviations**: HPV - Human Papillomavirus; HrHPV - high-risk HPV; HSIL - High-grade Squamous Intraepithelial Lesion; LSIL - Low-grade Squamous Intraepithelial Lesion

The two first groups were directly referred to a colposcopy unit, and 30 women (3.8% of all women who tested NILM) presented HSIL+ .

Discussion

According to WHO and HPV Action Network, cervical cancer screening remains an important tool for cervical cancer eradication worldwide [6,23]. Indeed, cervical cancer screening programs have contributed to reduce incidence and mortality, especially in developed countries [3,4]. For several decades, cytology based-testing was gold standard for cervical cancer screening, initially through a cervical smear and, more recently, using LBC [24]. Over the last decade, however, HrHPV genotyping-based cervical cancer screening demonstrated higher efficiency, including superior sensitivity and NPV, as well as amenability for high throughput [25]. Nonetheless, its lower specificity entails increased referral to colposcopy, as well as risk of overdiagnosis and consequent overtreatment. Herein, we assessed, for the first time, the impact of this screening strategy on the clinical outcome of women enrolled in the Cervical Cancer Screening Program of Northern Portugal.

The 2020 report for cervical cancer screening programs in Portugal disclosed a compliance rate of 85.5% for Northern Portugal [26], which is considered satisfactory. Furthermore, we found that 15% of the women tested positive for HrHPV, with 25% of them corresponding to multiple HrHPV infection, in line with our previous findings in a much larger dataset from this same program [19]. Moreover, the genotype frequency distribution found in this cross-sectional study also parallels the one previously published [19]. Thus, we may conclude that the cohort utilized in the present study is largely representative of the larger population from which it was extracted.

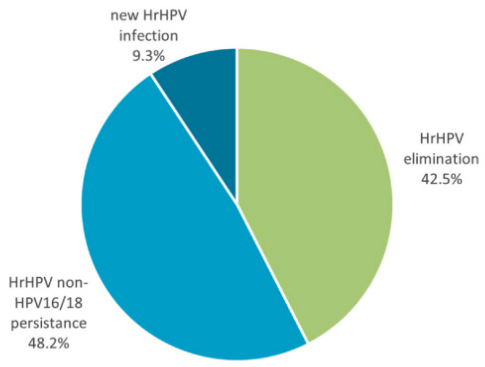

Fig. 4. – Distribution of HrHPV testing results among women which repeated sample collection after one year. Abbreviations: HPV – Human Papillomavirus; HrHPV – high risk HPV.

Our results underline one of the downsides of using first line HrHPV genotyping in for screening, more specifically, the high rate of colposcopy referral, negatively impacting in the activity of Cervical Pathology Units. Following European guidelines, many countries use cytology as triage test for HrHPV-positive women [10,24], but the ideal threshold for referral remains controversial [27]. Some European countries use ASC-US as threshold [28,29], whereas Australia set ASC-H [29–31]. Gultekin and colleagues reported a similar positive predictive value (PPV) using ASC-US or LSIL as threshold [32], although their findings have not been corroborated by others [28,33]. This discrepancy might be due to the subjectivity of cytology interpretation. Additional markers such as dual p16/Ki-67 staining have been proposed to overcome cytology subjectivity, although its value has not been fully established [34]. Interestingly, in our study, HSIL or worse lesions were observed in only 12% of women which performed colposcopy due to abnormal cytology result in the non-HPV16/18 HrHPV+ group (corresponding to 23% of all women with abnormal cytology regardless of HrHPV genotyping). Notwithstanding its limitations, cytology after partial HrHPV genotyping has been shown to be more cost-effective than cytology alone for triage of HrHPV-positive women [13,28,30,34]. Furthermore, some studies indicate that extended HrHPV genotyping might be helpful for triage of non-HPV16/18 HrHPV+ women with a cytology result of NILM [35] or ASC-US [36].

The potential for inappropriately high referral rate for colposcopy in our program might also derive from the use of HrHPV genotyping in women aged 25 to 30 years old. Indeed, it has been suggested that in this age group, first line cytology should be used to deal with the high prevalence of HrHPV infection which will mostly be cleared [14]. Although 30 years old has been recommended as the starting age for primary HrHPV genotyping-based screening, some studies have shown that HrHPV testing might detect a higher rate of cervical intraepithelial neoplasia (CIN)3 or worse lesions than cytology-based screening in women aged 25 years or older [9]. Thus, once again, more adequate triage strategies should be emphasized, enabling to better forecast persistence or clearance of HrHPV infection.

Interestingly, we found that more than 10% of cases directly referred to colposcopy were due to persistent non-HPV16/18 HrHPV infection. Although about 33% of cases achieved HrHPV clearance, more than 25% disclosed intraepithelial lesion, with 7% of cases referred depicting a diagnosis of HSIL or worse. The management of cases HrHPV-positive and cytology-negative constitute a challenge considering that their risk of developing a neoplastic lesion is higher than HrHPV-negative women, but compliance to re-screen and treatment is a key factor for the cervical cancer screening program's success. European guidelines recommend repeat HrHPV testing after at least 12 months [10], which was already proven to be more cost-

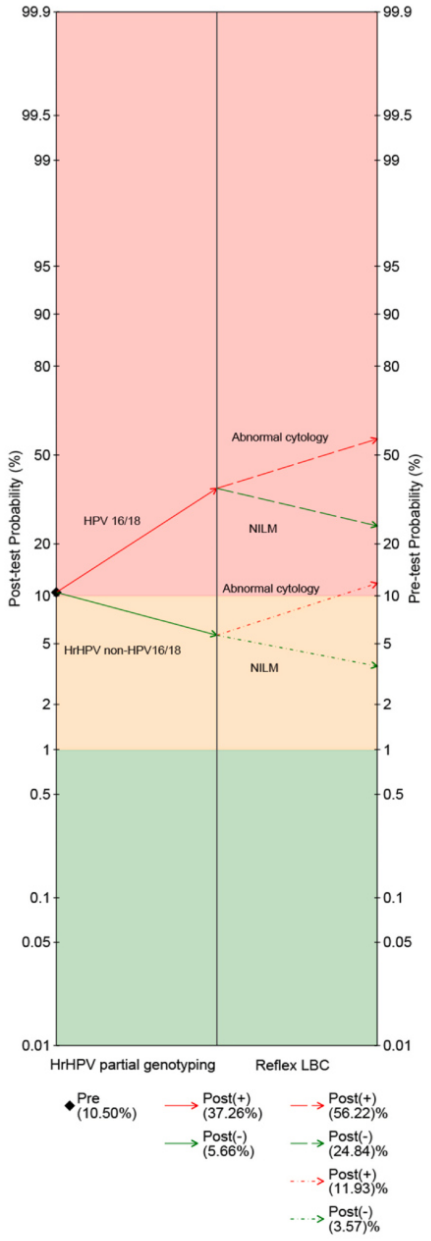

Fig. 5. – Pre-test and post-test probability (%) of high grade squamous intraepithelial lesion (HSIL) or worse. Abbreviations: HrHPV – high risk Human Papillomavirus; LBC – Liquid-based Cytology; NILM – Negative for Intraepithelial Lesion or Malignancy.

effective than at 24 months [37]. Furthermore, the attendance rate in the repeat test was reported to be lower than in the first-round screening among HrHPV-positive and cytology-negative women, further decreasing in subsequent rounds [37]. Intriguing, in our study, a high percentage (more than 25%) of women were lost to follow-up, which may be considered a limitation. Since we analyzed samples from January 2020, the follow-up period mostly occurred during COVID-19 pandemic, which may partially explain these results. Remarkably, our data are in line with a Danish study that reported a reduction in cervical cancer screening participation during COVID-19 pandemic [38]. Indeed, some women stated they did not attend cervical cancer screening during the COVID-19 pandemic due to the fear of contracting or transmitting COVID-19 [39]. Unfortunately, a disruption in cervical cancer screening programs occurred globally during COVID-19 [40], which we also witnessed in the Northern Portugal program during 2020 [26], although significant recovery was observed in the following year [41]. Nonetheless, we are aware that a proportion of those women were followed in the private sector, for which information was unavailable.

In accordance with published literature, we confirmed that HPV16/18 infection associates with higher risk of developing HSIL or worse compared to non-HPV16/18 HrHPV infection [28,30], emphasizing the significantly higher carcinogenic potential of those two genotypes. Partial genotyping of HrHPV as direct referral for colposcopy has been proven to be cost-effective in both vaccinated and unvaccinated women, which might lead to a reduction of cervical cancer incidence and mortality [15,30,37]. Although in unvaccinated cohorts partial genotyping might be associated with increased colposcopy referral, in vaccinated cohorts it might increase effectiveness owing to the high PPV [15,37]. Notwithstanding the substantially lower risk of non-HPV16/18 HrHPVs, the higher proportion of women infected with these genotypes prompts the need for adequate triage and follow-up, enabling the identification of cases more prone to evolve into HSIL and worse lesions. Importantly, our results emphasize that the high sensitivity of HrHPV testing has a substantial impact, especially for women with NILM. In line with our findings, several previous studies have reported a similar percentage of HSIL or worse among women with NILM results (only about 5%) [42], highlighting the gains of changing the screening program from first line cytology to HrHPV genotyping.

Overall, our study shows that compliance in the Cervical Cancer Screening Program of Northern Portugal is in line with the best practices reported in the literature. [11,14,37,43]. Indeed, RCCSP has a pipeline similar to that of some developed and developing countries in Europe [24]. The Turkish national screening program discloses a quite similar strategy to RCCSP [24] but the Turkish population has low HrHPV positivity (only around 4%) which is reflected in a lower number of women needing triage [44]. Interestingly, Gultekin and colleagues reported on the importance of a centralized lab for HrHPV and cytology testing for a high-quality performance [32].

Although our study concerned only one-month period of RCCSP and there was a lack of follow-up information for a proportion of women who tested positive for HrHPV, constituting the major limitations of the study, our results clearly demonstrate the strengths and weaknesses of RCCSP. This has a strong impact on the stakeholders involved in the program. Indeed, the significant increase in referral to colposcopy which occurred after implementation of first-line HrHPV testing translated into increased financial burden and a negative perception by the population on the access to care within the public health system.

The continuous improvements to perfect the strategy of colposcopy referral by means of improved PPV and increase screening participation are key to the success of this cervical cancer screening program. In this scenario, the emergence of new biomarkers for triage of HrHPV-positive women, including DNA methylation markers, holds the promise of further contributing to the sustainability of these programs [17]. Indeed, a recently published meta-analysis from our group demonstrated the adding value of DNA methylation-based biomarkers for triage of HrHPV-positive women [17]. These molecular biomarkers might constitute an effective alternative to cytology, also allowing for the expansion of programs based on self-

collected samples, in which cytology cannot be performed. Importantly, self-sampling has been shown to be cost-effective for cervical screening among vaccinated and unvaccinated women, holding the key to reach unscreened women [45].

Conclusion

In conclusion, this snapshot of the performance and mid-term clinical outcomes of the regional cervical cancer screening of Northern Portugal confirmed the reliability and effectiveness of first-line HrHPV genotyping, revealing the need for developing more effective triage tools, which is key to ensure the sustainability of cervical cancer screening programs, especially in the era of HrHPV vaccination.

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CRediT authorship contribution statement

SS prepared all the collected data, analyzed data, and drafted the manuscript (Investigation; Formal analysis; Writing - Original Draft). JPS assisted in data analysis (Formal analysis). AS and HS performed HrHPV testing in all samples and provided data from January of 2020 (Investigation). PM and IB supervised all the samples processing, reports, and data acquisition from the Cervical Cancer Screening Program of Northern Portugal, with the assistance of FT (Supervision). JB collected clinical follow-up data from Northern Portugal Cervical Cancer Screening Program samples (Investigation). RH and CJ designed and supervised the study and revised the manuscript (Conceptualization; Supervision; Funding acquisition; Project administration). All the authors read and approved the final version of the manuscript (Writing - Review & Editing).

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study protocol is part of a larger research project which was approved by the institutional review board of the Portuguese Oncology Institute of Porto (Comissão de Ética para a Saúde – CES-371/2017).

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2024.04.020.

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Supplementary material

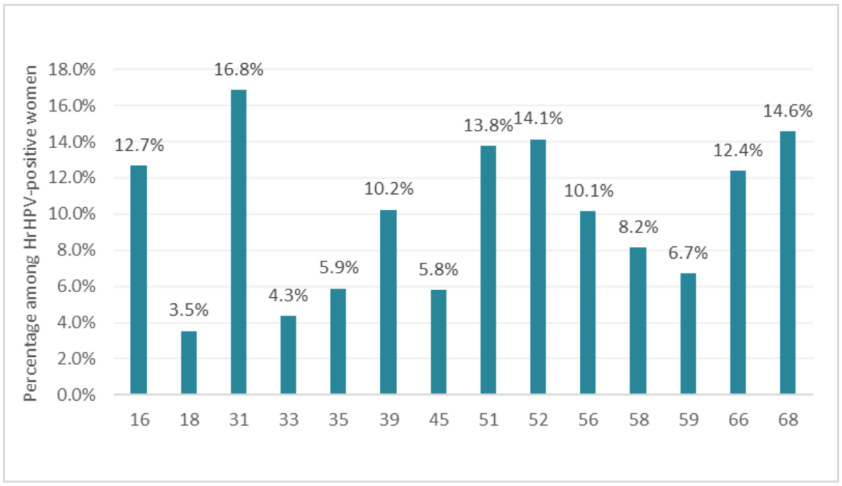
Available at https://ars.els-cdn.com/content/image/1-s2.0-S1876034124001333-mmc1.docx

Appendix A

Supplementary Material

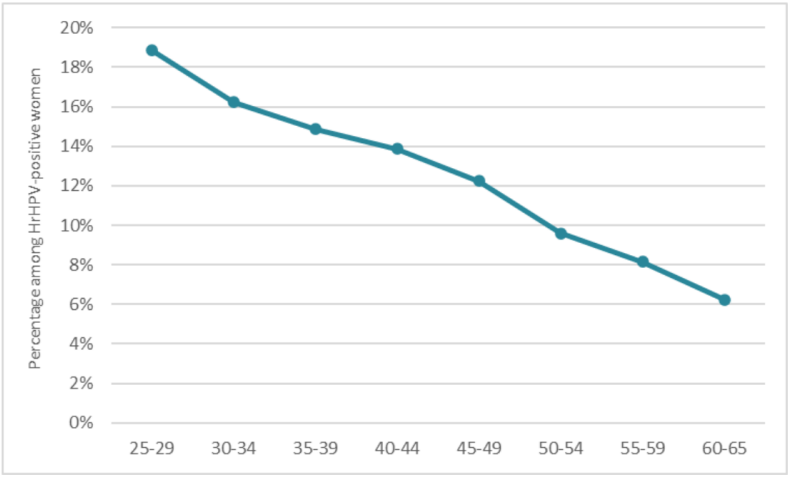
Supplementary Figures – Preliminary outcomes of the Cervical Cancer Screening Program of Northern Portugal: a snapshot

Supplementary Figure S1



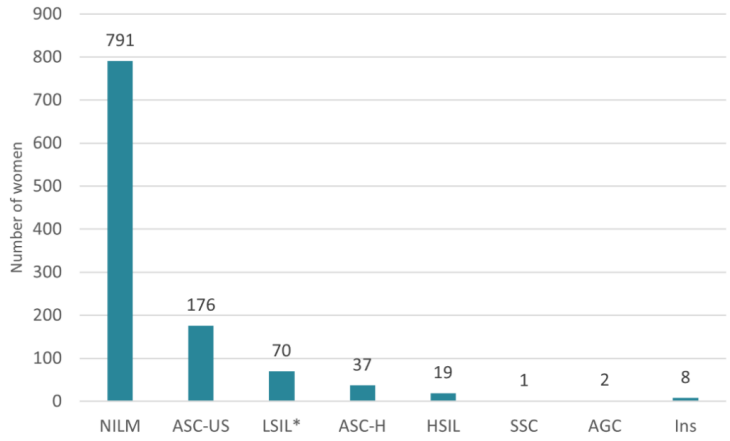
Supplementary Figure S1 – Prevalence of high-risk Human Papillomavirus (HrHPV) genotype in this study's cohort. Note: the sum of each HrHPV genotype cases percentage is higher than 100% due to the existence of cases with multiple HrHPV infections.

Supplementary Figure S2



Supplementary Figure S2 – Percentage of HrHPV-positive women according to age group. Abbreviations: HPV – Human Papillomavirus; HrHPV – high risk HPV.

Supplementary Figure S3



Supplementary Figure S3 – Distribution of cytology results. *1 case was classified as LSIL+ASC-H. Abbreviations: AGC – Atypical Glandular Cells; ASC-H – Atypical Squamous Cells – cannot exclude High grade squamous intraepithelial lesion; ASC-US – Atypical Squamous Cells of Undetermined Significance; HSIL – High-grade Squamous intraepithelial lesions; LSIL – Low-grade Squamous intraepithelial lesions; NILM – Negative for Intraepithelial Lesion Malignancy; SSC – Squamous cell carcinoma.

Supplementary Tables – Preliminary outcomes of the Cervical Cancer Screening Program of Northern Portugal: a snapshot

Table S1 - High risk Human Papillomavirus genotype prevalence according age groups

Age Group	**25-29**	**30-34**	**35-39**	**40-44**	**45-49**	**50-54**	**55-59**	**60-65**	**Total**
Total, n (%)	887 (12.19%)	832 (11.43%)	922 (12.67%)	1037 (14.25%)	1066 (14.65%)	958 (13.16%)	906 (12.45%)	670 (9.20%)	7278 (100%)
HrHPV Negative n	678	652	758	880	931	852	815	600	6166
HrHPV Positive n	208	179	164	153	135	106	90	69	1104
HPV-16	10	33	16	23	22	13	13	10	140
HPV-18	2	7	5	10	7	3	4	1	39
HPV-31	25	27	41	26	22	17	14	14	186
HPV-33	7	8	4	12	4	7	3	3	48
HPV-35	12	6	11	11	6	7	8	4	65
HPV-39	35	9	22	12	16	8	4	7	113
HPV-45	9	7	13	8	8	8	8	3	64
HPV-51	37	26	21	21	15	18	8	6	152
HPV-52	41	26	19	19	16	14	12	9	156
HPV-56	25	18	12	13	18	10	9	7	112
HPV-58	20	18	8	13	8	8	10	5	90
HPV-59	22	10	12	7	7	9	5	2	74
HPV-66	40	17	23	19	11	11	11	5	137
HPV-68	28	25	27	18	17	19	15	12	161
Multiple infections	72	40	48	42	26	32	22	13	295
Unsatisfactory/ Insufficient n (%)	1	1	0	4	0	0	1	1	8

Abbreviations: HPV - Human Papillomavirus; HrHPV - high-risk HPV; n - number.

Table S2 – Number of women, pre-test and post-test probability

	Women (n)	HSIL or worse (n)	Pre-test probability	Post-test probability
HrHPV	1104	116		
HPV 16/18	169	63	0.105	0.347
Abnormal Cytology	72	43	0.347	0.429
NILM	96	20	0.347	0.162
non-HPV16/18	935	53	0.105	0.051
Abnormal Cytology	233	28	0.051	0.103
NILM	695	25	0.051	0.030

Abbreviations: HPV - Human Papillomavirus; hrHPV - high-risk HPV; HSIL - High-grade Squamous Intraepithelial Lesion; LSIL - Low-grade Squamous Intraepithelial Lesion; n - number; NILM - Negative for Intraepithelial Lesion or Malignancy.

Table S3 – Outcomes of women who tested Negative for Intraepithelial Lesion or Malignancy n=791

Outcome	HPV16/18 (n= 96)	HPV persistent (n=136)	Women sent to screening repetition (n=559)
HSIL or worse (n=45)	20	10	15
Normal or LSIL (n=559)	67	116	396
Lost to follow-up (n=167)	9	10	148

Abbreviations: HPV - Human Papillomavirus; HrHPV - high-risk HPV; HSIL - High-grade Squamous Intraepithelial Lesion; LSIL - Low-grade Squamous Intraepithelial Lesion; n - number.

CHAPTER IV –
Research Paper II

Research Paper II

Paper II – DNA methylation as a triage marker for colposcopy referral in HPV-based cervical cancer screening: a systematic review and meta-analysis

Clinical Epigenetics (2023) 15:125
DOI: 10.1186/s13148-023-01537-2

Sofia Salta, João Lobo, Bruno Magalhães, Rui Henrique* and Carmen Jerónimo*

Author's personal contribution:

Literature search, selection and data extraction, statistical analysis performance and interpretation of the results, and draft of the manuscript.

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Clinical Epigenetics

RESEARCH Open Access

DNA methylation as a triage marker for colposcopy referral in HPV-based cervical cancer screening: a systematic review and meta-analysis

Sofia Salta[1,2,3], João Lobo[1,4,5], Bruno Magalhães[6,7,8], Rui Henrique[1,4,5*†] and Carmen Jerónimo[1,5*†]

Abstract

Background Screening plays a key role in secondary prevention of cervical cancer. High-risk human papillomavirus (hrHPV) testing, a highly sensitive test but with limited specificity, has become the gold standard frontline for screening programs. Thus, the importance of effective triage strategies, including DNA methylation markers, has been emphasized. Despite the potential reported in individual studies, methylation markers still require validation before being recommended for clinical practice. This systematic review and meta-analysis aimed to evaluate the performance of DNA methylation-based biomarkers for detecting high-grade intraepithelial lesions (HSIL) in hrHPV-positive women.

Methods Hence, PubMed, Scopus, and Cochrane databases were searched for studies that assessed methylation in hrHPV-positive women in cervical scrapes. Histologically confirmed HSIL was used as endpoint and QUADAS-2 tool enabled assessment of study quality. A bivariate random-effect model was employed to pool the estimated sensitivity and specificity as well as positive (PPV) and negative (NPV) predictive values.

Results Twenty-three studies were included in this meta-analysis, from which cohort and referral population-based studies corresponded to nearly 65%. Most of the women analyzed were Dutch, and *CADM1, FAM19A4, MAL,* and *miR124-2* were the most studied genes. Pooled sensitivity and specificity were 0.68 (CI 95% 0.63–0.72) and 0.75 (CI 95% 0.71–0.80) for cervical intraepithelial neoplasia (CIN) 2+ detection, respectively. For CIN3+ detection, pooled sensitivity and specificity were 0.78 (CI 95% 0.74–0.82) and 0.74 (CI 95% 0.69–0.78), respectively. For pooled prevalence, PPV for CIN2+ and CIN3+ detection were 0.514 and 0.392, respectively. Furthermore, NPV for CIN2+ and CIN3+ detection were 0.857 and 0.938, respectively.

Conclusions This meta-analysis confirmed the great potential of DNA methylation-based biomarkers as triage tool for hrHPV-positive women in cervical cancer screening. Standardization and improved validation are, however, required. Nevertheless, these markers might represent an excellent alternative to cytology and genotyping for colposcopy referral of hrHPV-positive women, allowing for more cost-effective screening programs.

†Rui Henrique and Carmen Jerónimo are Joint Senior.

*Correspondence:
Rui Henrique
henrique@ipoporto.min-saude.pt
Carmen Jerónimo
carmenjeronimo@ipoporto.min-saude.pt
Full list of author information is available at the end of the article

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Keywords Cervical cancer screening, Triage, DNA methylation, Biomarkers, Colposcopy referral

Background

Currently, cervical cancer remains a significant public health concern at global level. Not only does it represent the fourth most incident malignancy in women (with an age-standardized incidence rate of 13.3 per 100,000 female individuals in 2020, worldwide), but also it is the third most deadly cancer (with an age-standardized mortality rate of 7.3 per 100,000 women in 2020 worldwide) [1]. These figures, nonetheless, hide remarkable geographical differences, with cervical cancer-related deaths being more impressive in countries with low human development index [1]. Although this may be partially explained by limited access to high-quality medical care, lack of effective preventive strategies, including screening, constitutes the major cause. Because cervical cancer is a preventable disease, screening strategies, based on cervical cytology and/or high-risk HPV (hrHPV) testing implemented at younger ages (below 30–35 years), detect with noticeable sensitivity and specificity the precancerous lesions amenable for treatment before overtly invasive cancer develops [2, 3]. The vast majority of cervical cancers are hrHPV-related, and the implication of this virus in cervical cancer pathobiology is well known, namely its effect on the transformation of epithelial surfaces like the squamous–columnar junction of the cervix or the lymph epithelium of the base of tongue and tonsils [4]. In recent years, screening strategies have progressively focused on hrHPV testing as first-line screening test [5–7], owing to its higher sensitivity. However, hrHPV infections detected may also correspond to transient infection, and thus, this test is unable to specifically identify women which really need to be referred for a specialized consultation and undergo colposcopy-guided biopsy, a rather invasive procedure. Indeed, an accurate test which might identify clinically relevant hrHPV infections is key to reduce the number of unneeded referrals and interventions (with associated risks and costs) as well as hrHPV test repetitions [8, 9]. DNA methylation, the most studied epigenetic mechanism involved in gene expression regulation, has been successfully explored as a source of noninvasive disease biomarkers [10, 11]. Specifically, in cervical cancer, shifts in promoter methylation levels of several genes (both human and part of the HPV genome) have been associated with HPV status, lesion progression, and patient outcome [10, 11]. Despite a very promising performance demonstrated in individual studies, the fact is that such methylation-based tests have not moved from research to clinical practice, yet. Importantly, reports in the literature are characterized by heterogeneity of study settings, populations, methodological strategies, technicalities, and cutoff values used, among other variables, hampering a comprehensive overview of these tests' performance and their real clinical usefulness, as well as the added value of comparing with standard methods like cytology and hrHPV genotyping [3].

In this systematic review and meta-analysis, we aimed to evaluate the performance of DNA methylation-based biomarkers for detecting high-grade intraepithelial lesions (HSIL), i.e., cervical intraepithelial neoplasia (CIN)2 + and CIN3 + in hrHPV-positive women and assess their potential as triage biomarkers in these women, to better ascertain their value in the context of cervical cancer screening. Furthermore, we identified the gaps that still preclude their translation into the clinics.

Results

Literature overview

Our search retrieved 536 records in PubMed, 498 in Scopus, and 27 in Cochrane, achieving a total of 1061 records, 73 of which were duplicates (Fig. 1). From the remaining 988 publications, 852 were excluded after abstract and title review. Another 113 publications were further excluded: 11 did not use cervical smears/scrapes; 71 did not perform methylation analysis in a hrHPV-positive women setting (i.e., as triage); 8 studies did not test hrHPV in the samples used; 14 only presented hrHPV methylation; 4 performed DNA methylation analysis in only a subset of samples (e.g., CIN1 vs. CIN3); 4 did not allow for data extraction; and 1 disclosed a high level of overlap with another included study (B1) [12]. Hence, 23 articles were included in the final analysis, and these are summarized in Table 1 and Additional file 1: Table S1.

Among these studies, one analyzed a population from 4 different countries and was thus considered as representing four independent studies [13], and two studies reported two sets of different samples, which were also considered independent [14, 15]. Although most of the studies (12/23) were conducted in the Dutch population [12–23], one also analyzed populations from Scotland, Denmark, and Slovenia [13]. Four additional studies were conducted in European women [24–27], while 5 studies were conducted in the Chinese population [28–32], one in the Canadian population [34], and another in the Kenyan population [33].

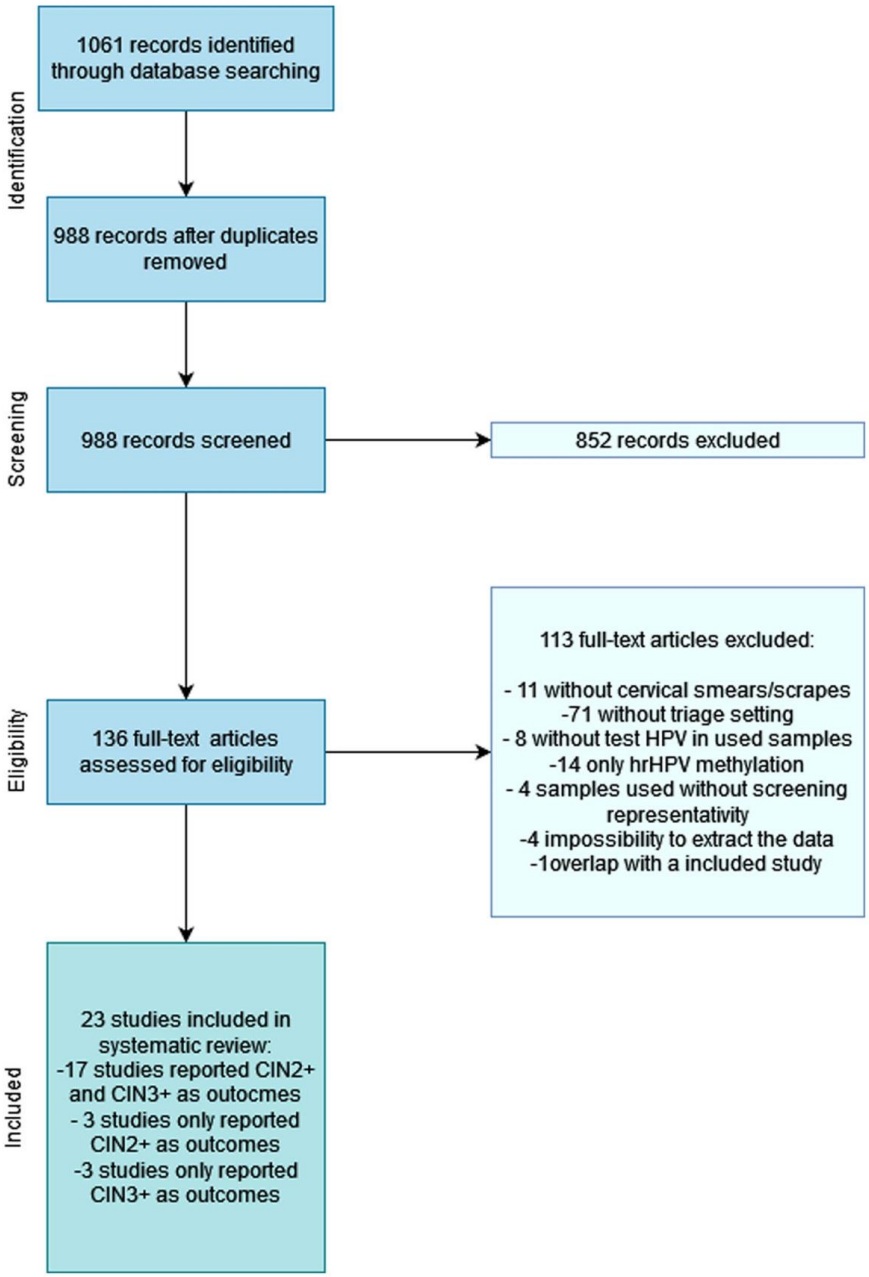

Fig. 1 Flowchart of the systematic review and studies included in the meta-analysis

Eight articles evaluated DNA methylation of *cell adhesion molecule 1* (*CADM1*), *TAFA chemokine-like family member 4* (*TAFA4*, also known as *FAM19A4*), *mal, T cell differentiation protein* (*MAL*), and *microRNA 124-2* (*miR124-2*) genes in different combinations [12–14, 20–23, 33], whereas four studies only

Table 1 Individual studies characterization

ID	Author	Year of study publication	Type of study	Study population origin (country)	Cohort origin	Sample type	Age (range)	Number of samples	Number of CIN2+ (%)	Number of CIN3+ (%)	DNA methylation markers studied	Panel versus single versus both	Platform	Pre-established cutoff yes/no/unclear	Refs.
Studies with health professional-collected samples															
L1	Bonde [a]	2021	Convenience[1]	Scotland	PAVDAG	Cervical scrape	30–61	161	40 (24.8%)	25 (15.5%)	FAM19A4/miR124-2	Panel	QMSP	Yes	[13]
	Bonde [b]			Denmark	VALGENT4 + routine screening		30–65	424	75 (17.7%)	57 (13.4%)					
	Bonde [c]			Slovenia	Routine screening		30–76.3	928	102 (11.0%)	60 (6.5%)					
	Bonde [d]			The Netherlands	VUSA screen		29–61	871	155 (17.8%)	106 (12.2%)					
B1	Hesselink	2011	Convenience[1]	The Netherlands	Routine screening	Cervical scrape	19–62	236	58 (24.6%)	38 (16.1%)	CADM1/MAL	Panel	QMSP	Yes	[12]
F5	Verhoef	2015	Referral population based	The Netherlands	PROHTECT-3 (Cytology arm)	Cervical scrape	38–48	364	90 (24.7%)	62 (17.0%)	CADM1/MAL	Panel	QMSP	Yes	[20]
L2	Vink	2021	Referral population based	The Netherlands	POBASCAM	Cervical scrape	26–61	979	–	115 (11.7%)	FAM19A4/miR124-2	Panel	QMSP	Yes	[23]
F1	Vuyst	2015	Cohort	Kenya	HIV-infected	Cervical scrape	18–55	248	93 (37.5%)	–	CADM1/MAL/miR124-2	Panel	QMSP	No	[33]
I1	Bu	2018	Cohort	China	Routine screening	Cervical scrape	–	215	118 (54.9%)	–	FAM19A4	Single	QMSP	No	[28]
E4	De Strooper	2014	Cohort	The Netherlands	Routine screening	Cervical scrape	19–62	218	52 (23.9%)	33 (15.1%)	FAM19A4	Single	QMSP	Yes	[17]
G4	Luttmer	2016	Referral population based	The Netherlands	COMETH	Cervical scrape	18–70	508	180 (35.4%)	90 (17.7%)	FAM19A4	Single	QMSP	Yes	[18]
J1	Cook	2019	Case–control	Canada	FOCAL	Cervical scrape	25–65	257	107 (41.6%)	44 (17.1%)	S5 (EPB41L3/HPV16L1/HPV16L2/HPV18L2/HPV31L1/HPV33L2)	Panel	QMSP	Yes	[34]

Table 1 (continued)

ID	Author	Year of study publication	Type of study	Study population origin (country)	Cohort origin	Sample type	Age (range)	Number of samples	Number of CIN2+ (%)	Number of CIN3+ (%)	DNA methylation markers studied	Panel versus single versus both	Platform	Pre-established cutoff yes/no/unclear	Refs.
G2	Lorincz	2016	Case–control	United Kingdom	Predictors 3 (P3)	Cervical scrape	–	341	39 (11.4%)	–	S5 (EPB41L3/HPV16L1/HPV16L2/HPV18L2/HPV31L1/HPV33L2) and S4 (EPB41L3/HPV16L1/HPV16L2/HPV18L2/HPV31L1)	Panel	Pyrosequencing	Yes	[25]
E1	Boers	2014	Convenience[1]	The Netherlands	PROHTECT-3B	Cervical scrape	33–67	128	49 (38.3%)	34 (26.6%)	C13ORF18/EPB41L3/JAM3/TERT	Single	QMSP	No	[16]
K3	Li	2020	Cohorts	Chinese	Outpatient clinic population	Cervical scrape	18–67	227	129 (56.8%)	83 (36.6%)	ANKRD18CP/C13orf18/EPB41L3/JAM3/SOX1/ZCAN1	Both	QMSP	Yes	[29]
J7	van Leeuwen	2019	Referral population based	Slovenian	Slovenian HPV Prevalence Study	Cervical scrape	–	235	35 (14.9%)	19 (8.1%)	ANKRD18CP/C13orf18/EPB41L3/JAM3/SOX1/ZCAN1	Both	QMSP	Yes	[27]
H7	Yuan	2017	Case–control	China	Hospital-based study	Cervical scrape	–	259	164 (63.3%)	118 (45.6%)	C13ORF18/JAM3/SLIT2/SOX1/TERT	Both	Pyrosequencing	Unclear	[32]
F6	Yin	2015	Cohort	China	Outpatient clinic population	Cervical scrape	–	168	72 (42.9%)	31 (18.5%)	JAM3	Single	QMSP	No	[31]
E5	Hansel	2014	Cohort	Germany	Outpatient clinic population	Cervical scrape	18–81	217	84 (38.7%)	42 (19.4%)	DLX1/ITGA4/RXFP3/SOX17/ZNF671	Panel	QMSP	No	[24]

Table 1 (continued)

ID	Author	Year of study publication	Type of study	Study population origin (country)	Cohort origin	Sample type	Age (range)	Number of samples	Number of CIN2+ (%)	Number of CIN3+ (%)	DNA methylation markers studied	Panel versus single versus both	Platform	Pre-established cutoff yes/no/unclear	Refs.
H4	Schmitz	2017	Cohort	Germany	Outpatient clinic population	Cervical scrape	–	189	–-	89 (47.1%)	ASTN1/DLX1/ITGA4/RXFP3/SOX17/ZNF671	Panel	QMSP	Yes	[26]
H5	Tian	2017	Cohort	China	Outpatient clinic population	Cervical scrape	–	312	–-	155 (49.7%)	ZNF582/PAX1	Both[2]	QMSP	Yes	[30]
Studies with self-collected samples															
G1	De Strooper [a]	2016	Referral population based	The Netherlands	PROHTECT-3A	Self-collected cervicovaginal lavage	33–63	389	119 (30.6%)	78 (20.1%)	FAM19A4/miR124-2	Panel	QMSP	Yes	[14]
	De Strooper [b]	2016		The Netherlands	PROHTECT-2	Self-collected vaginal brush	30–62	254	99 (39.0%)	72 (28.3%)					
E10	Verhoef	2014	Referral population based	The Netherlands	PROHTECT-3 (methylation arm)	Self-collected cervicovaginal lavage	30–60	408	124 (30.4%)	79 (19.4%)	MAL/miR124-2	Panel	QMSP	Yes	[21]
E9	Verhoef	2014	Referral population based	The Netherlands	PROHTECT-3	Self-collected cervicovaginal lavage	30–64	1019	225 (22.1%)	147 (14.4%)	MAL/miR124-2	Panel	QMSP	Yes	[22]
G3	Luttmer	2016	Referral population based	The Netherlands	COMETH	Self-collected cervicovaginal lavage	18–66	450	153 (34.0%)	75 (16.7%)	FAM19A4	Single	QMSP	Yes	[19]

Table 1 (continued)

ID	Author	Year of study publication	Type of study	Study population origin (country)	Cohort origin	Sample type	Age (range)	Number of samples	Number of CIN2+ (%)	Number of CIN3+ (%)	DNA methylation markers studied	Panel versus single versus both	Platform	Pre-established cutoff yes/no/unclear	Refs.
I8	Verlaat[a]	2018	Convenience[1]	The Netherlands	PROHTECT-3B	Self-collected vaginal brush	27–75	287	109 (38.0%)	81 (28.2%)	ASCL1/LHX8/ST6GALNAC5	Panel	QMSP	Yes	[15]
	Verlaat[b]	2018		The Netherlands	PROHTECT-3	Self-collected cervico-vaginal lavage	33–63	199	65 (32.7%)	43 (21.6%)					

[1] The study used a selection of samples from a previous population-based study; [2]ZNF582 was evaluated individually and as panel with PAX1

evaluated *FAM19A4* methylation [17–19, 28]. Two studies assessed the performance of the S5 classifier [a methylation panel comprising *erythrocyte membrane protein band 4.1 like 3* (*EPB41L3*), *HPV16L1, HPV16L2, HPV18L2, HPV31L1, HPV33L2*], one of which also included the evaluation of S4 classifier (*EPB41L3, HPV16L1, HPV16L2, HPV18L2, HPV31L1*) [25, 34]. *EPBL41L3* methylation levels were assessed in three more studies alone, and different combinations with *ankyrin repeat domain 18C, pseudogene* (*ANKRD18CP*), *rubicon-like autophagy enhancer* (*RUBCNL* also known as *C13orf18*), *junctional adhesion molecule 3* (*JAM3), SRY-box transcription factor 1* (*SOX1*), *telomerase reverse transcriptase* (*TERT*), *zinc finger and SCAN domain containing* 1 (*ZCAN1*) [16, 27, 29]. *C13orf18, JAM3, SOX1,* and *TERT* were evaluated individually and in panels with different combinations of two genes, including *slit guidance ligand 2* (*SLIT2*) gene [32]. One study evaluated *JAM3* methylation individually [31]. Two more studies focused on methylation of *distal-less homeobox 1* (*DLX1*), *integrin subunit alpha 4* (ITGA4), *relaxin family peptide receptor 3* (*RXFP3*), *SRY-box transcription factor 17* (*SOX17*), and *zinc finger protein 671* (*ZNF671*), and other also evaluated the methylation of *ANKRD18CP*. Both studies assessed the genes individually and as a panel [24, 26]. Furthermore, one study reported methylation levels of *zinc finger protein 582* (*ZNF582*) individually and as part of a panel with *paired box 1* (*PAX1*) [30]. Lastly, one study assessed the methylation levels of *achaete-scute family bHLH transcription factor 1* (*ASCL1*), *LIM homeobox 8* (*LHX8*), and *ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5* (*ST6GALNAC5*) in a panel [15].

Overall, 17 (74%) studies reported DNA methylation markers performance for both outcomes considered in this systematic review and meta-analysis (CIN2+ and CIN3+) [12–22, 24, 27, 29, 31, 32, 34]. Three (13%) studies only reported DNA methylation-based markers performance for CIN2+ outcome [25, 28, 33], and three (13%) more only reported DNA methylation-based markers performance for CIN3+ outcome [23, 26, 30]. Of note, 5 (22%) studies were conducted in self-collected samples [14, 15, 19, 21, 22]. Moreover, eight studies (35%) were referral population-based studies [14, 18–23, 27], eight studies (35%) were cohort studies [17, 24, 26, 28–31, 33], three (13%) were case–control studies [25, 32, 34], and four (17%) were convenience studies [12, 13, 15, 16].

Concerning methylation cutoffs, seventy-three percent of the studies disclosed a predefined cutoff for positivity [12–15, 17–23, 25–30, 34], mostly established through receiver operating characteristics (ROC) curve analysis in a training set or previous studies.

Quality assessment

The quality of individual studies was assessed using QUADAS-2 and is summarized in Fig. 2 and Additional file 1: Table S2. The primary source of bias was patient selection. About 20% showed high patient selection bias, which was mainly associated with the design of the primary study. Nonetheless, the triage setting reported by some studies as a secondary outcome might also have led to bias.

Moreover, studies with high patient selection bias were associated with an enrichment of CIN2+ and or CIN3+ lesions, thus not representing a real population-based scenario. Most studies did not fully describe the women lost for follow-up or the interval between the methylation test and the reference test. Studies with high selection bias were removed from some of the analyses to evaluate the impact on biomarker performance ($n=7$).

Diagnostic accuracy in an hrHPV-positive women triage setting

Diagnostic performance of the reported methylation markers was assessed, and the main performance indicators are depicted in Table 2. For both outcomes, CIN2+ and CIN3+, sensitivity, specificity, and summary receiver operating characteristic (SROC) curve (Fig. 3) were pooled for: i) all the markers reported in all studies; ii) the best markers reported in each study (avoiding considering the same sample more than once); iii) the most frequently studied genes (*CADM1, FAM19A4, MAL* and *miR124-2*); and iv) studies which set the threshold to achieve 70% specificity. For CIN2+ detection, the pooled AUC was above 73% in all analysis models. Additionally, to evaluate the impact of bias, for analysis ii), high-bias studies were removed, and the outcomes were predicted (Table 2).

Considering only one entrance for the study, sensitivity and specificity for CIN2+ detection were 68% (CI 95% 63–72%) and 75% (CI 95% 71–80%), respectively. Furthermore, for CIN3+ detection, the pooled AUC was higher than 77% in all groups. For the best markers of each study, sensitivity reached 78% (CI 95% 74–82%), and 74% (CI 95% 69–78%) specificity was achieved. The pooled sensitivity and specificity of each study are further represented as a forest plot in Additional file 2: Figs. S1 and S2. When studies were stratified by study type, sensitivity and specificity were similar (Table 3).

The same was observed after excluding high-bias studies from the analysis (Table 2). Moreover, all analyzed models, except for model iv for CIN3+ detection, were associated with high and significant heterogeneity (Q

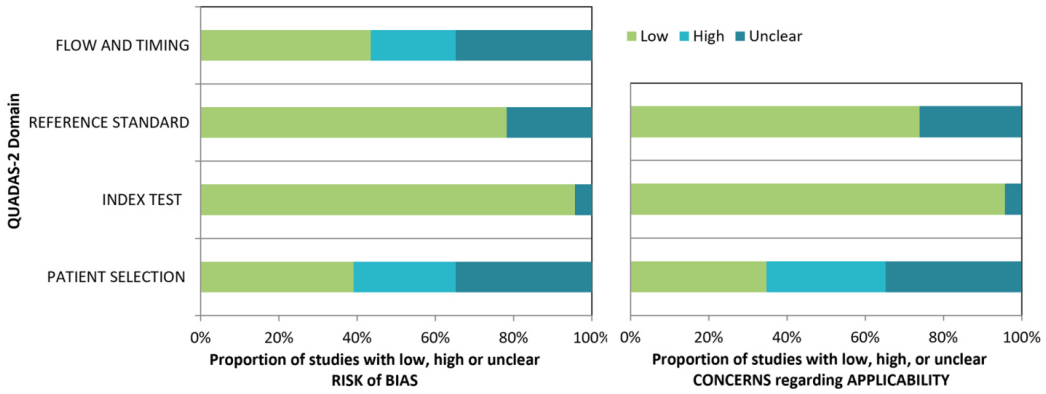

Fig. 2 Quality assessment of the individual studies using QUADAS-2 tool. The left panel depicts the risk of bias of the studies and the right panel the risk of concerns regarding applicability

with *p-value* ≤ 0.051 and I^2 above 89%). A univariable meta-regression showed a significant correlation with cohort overlapping among studies, the use of one methylation panel, and the existence of a predefined cutoff (Additional file 2: Fig. S3) for both CIN2+ and CIN3 detection. Concerning CIN3+ detection, the type of sample (self-collected or not) was also found significant. When separately analyzed, studies with self-collected samples disclosed slightly lower performance for CIN 2+ [sensitivity: 63% (CI 95% 54–72%) vs. 70% (CI 95% 65–74%); specificity: 73% (CI 95% 63–81%) vs 76% (CI 95% 71–81%); AUC: 73% (CI 95% 69–77%) vs. 79% (CI 95% 75–82%)] and CIN3+ detection [sensitivity: 72% (CI 95% 63–80%) vs. 81% (CI 95% 76–85%); specificity: 70% (CI 95% 60–78%) vs. 75% (CI 95% 70–79%); AUC: 77% (CI 95% 74–81%) vs. 85% (CI 95% 82–88%)], comparatively to studies in which samples were collected by health professionals (Table 4).

The PPV and NPV values were calculated for all conditions reported above and are displayed in Table 5 and Additional file 2: Fig. S4, according to different prevalence ranges. For CIN2+ detection, the pooled prevalence varied between 25 and 35%, with a PPV between 0.446 and 0.620, and NPV always above 0.80. For a 30% prevalence, PPV ranged from 0.490 to 0.565, with an NPV remaining above 0.80. For CIN3+ detection, the PPV for the pooled prevalence ranged between 0.294 and 0.515, with an NPV above 90% for all conditions. For 20% prevalence, PPV was above 0.87 and NPV above 0.90.

Discussion

Primary prevention of cervical cancer with a vaccine against hrHPV was an important step for hrHPV control and eradication. Nonetheless, secondary prevention still plays a crucial role in the reduction of incidence and mortality of cervical cancer, especially among unvaccinated women [35, 36]. First-line hrHPV testing for cervical cancer screening became the gold standard for many European countries at regional or national levels, as proposed by HPV Action Network from European Cancer Organisation in *Viral Protection: Achieving the Possible. A Four-Step Plan for Eliminating HPV Cancers in Europe* [7]. However, this shift challenges the sustainability of screening programs, as the number of women referred to colposcopy significantly increased, mostly due to the limited specificity of hrHPV testing, with the identification of transient infection, and the failure to discriminate lesions with risk of progression from those in regression [37, 38].

In recent years, DNA methylation-based biomarkers have been investigated as potential tools for triage of hrHPV-positive cases, in an attempt to reduce the number of cases referred to colposcopy, avoiding overdiagnosis and consequent overtreatment. However, the evidence supporting the use of triage tests remains limited, as acknowledged by the latest Word Health Organization recommendations [39]. Therefore, we conducted a systematic review with a meta-analysis to better understand the actual value of these DNA methylation-based biomarkers.

One of the major challenges (and limitations) of this meta-analysis results from the fact that distinct markers alone or combined in several panel have been reported along with different methodologies (including genes studied and methodological approach), with only a very small number of studies having used exactly the same protocols. Additionally, in some studies a histological biopsy was not performed when the co-test was

Table 2 Meta-analysis of the performance of DNA methylation assays for the detection of CIN2+ and CIN3+

	N datasets	N samples	Pooled prevalence	Pooled Sensitivity, (95% CI)	Pooled Specificity, (95% CI)	Q (*p-value*)	I2 (95% CI)	Pooled AUC, (95% CI)
CIN2 + detection								
All studies with all markers [a,b][12–22, 24, 25, 27–29, 31–34]	55	16,022	0.35	0.67 (0.63–0.70)	0.80 (0.75–0.83)	422.771 ($p < 0.001$)	100 (99–100)	0.77 (0.73–0.81)
All studies with best markers [a,c][12–22, 24, 25, 27–29, 31–34]	25	9011	0.28	0.68 (0.63–0.72)	0.75 (0.71–0.80)	160.418 ($p < 0.001$)	99 (98–99)	0.77 (0.73–0.81)
Studies with *CADM1, FAM19A4, MAL* and *miR124-2*[a][12–14, 17–22, 28, 33]	15	6693	0.25	0.64 (0.59–0.68)	0.74 (0.69–0.78)	77.518 ($p < 0.001$)	97 (96–99)	0.74 (0.70–0.77)
Set threshold to achieve 70% specificity[12, 14, 17–20, 22, 33]	9	3686	0.29	0.59 (0.53–0.65)	0.74 (0.70–0.77)	17.604($p < 0.001$)	89 (77–100)	0.73 (0.69–0.77)
All studies with best markers excluding high-bias studies [a,d][12–15, 17–22, 24, 25, 27, 29]	20	7736	0.25	0.66 (0.61–0.70)	0.74 (0.69–0.78)	110.195 ($p < 0.001$)	98 (97–99)	0.75 (0.71- 0.79)
CIN3 + detection								
All studies with all markers [a,b][12–24, 26, 27, 29–32, 34]	55	16,669	0.24	0.78 (0.75–0.81)	0.77 (0.73–0.80)	353.267($p < 0.001$)	99 (99–100)	0.85 (0.81–0.87)
All studies with best markers [a,e][12–24, 26, 27, 29–32, 34]	25	9682	0.18	0.78 (0.74–0.82)	0.74 (0.69–0.78)	87.682 ($p < 0.001$)	98 (96–99)	0.83 (0.79 -0.86)
Studies with *CADM1, FAM19A4, MAL* and *miR124-2*[a] [12–14, 17–23]	14	7202	0.14	0.72 (0.68–0.76)	0.72 (0.67–0.76)	24.008 ($p < 0.001$)	92 (84–99)	0.77 (0.74–0.81)
Set threshold to achieve 70% specificity [12, 14, 17–20, 22, 33]	9	3655	0.17	0.69 (0.63–0.74)	0.74 (0.70–0.77)	2.778 ($p = 0.125$)	28 (0–100)	0.77 (0.73–0.80)
All studies with best markers excluding high-bias studies [a,f][12–15, 17–24, 27, 29, 30]	20	8681	0.17	0.77 (0.72–0.81)	0.73 (0.68–0.76)	58.123 ($p < 0.001$)	97 (94–99)	0.81 (0.77- 0.84)

[a] The sensitivity and specificity were estimated as reported by authors. When multiple thresholds reported, 70% specificity was selected. [b]Pooled together all the genes and gene combination reported in each study. For studies [16, 23, 25, 26, 29, 32], more than one entrance was considered. [c]Only one entrance per study was considered. The best combination reported by the authors was selected. For [16] and [32] was considered *JAM3*; for [29] and [27] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier (*EPB41L3/HPV16L1/HPV16L2/HPV18L2/HPV31L1/HPV33L2*). [d]Only one entrance per study was considered. The best combination reported by the authors was selected. For [29] and [27] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier (*EPB41L3/HPV16L1/HPV16L2/HPV18L2/HPV31L1/HPV33L2*). [e]Only one entrance for study was considered. The best combination reported by the authors was selected. For [30] was considered *PAX1/ZNF582*; for [16] was considered *JAM3*; for [29] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier; for [27] was considered *SOX1/ZSCAN1*; for [32] was considered *SOX1*. [f] Only one entrance for study was considered. The best combination reported by the authors was selected. For [30] was considered *PAX1/ZNF582*; for [29] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier; for [27] was considered *SOX1/ZSCAN1*; for [32] was considered *SOX1*

negative, which might be a source of bias, although the reported risk of misclassification is rather low [40, 41]. Furthermore, some studies considered the women lost to follow-up as negative for CIN2+ lesion, which might be associated with lesion misclassification and, therefore, might have impacted in the estimated sensitivity and specificity.

Notwithstanding, focusing on the best markers reported from each study, DNA methylation markers reached, overall, a specificity similar to that reported for

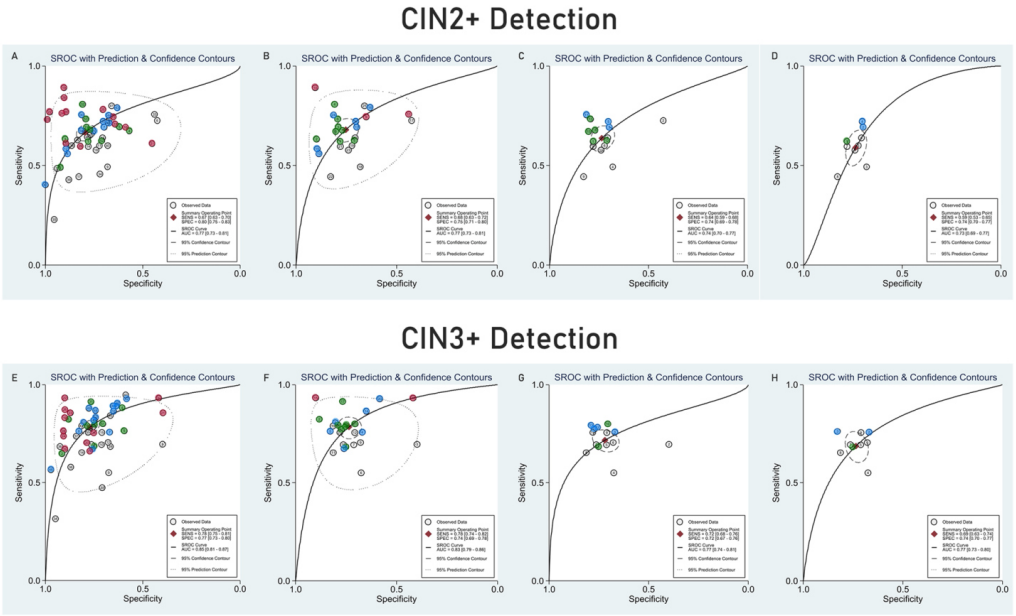

Fig. 3 SROC graphs for CIN 2 + (**A**–**D**) and CIN3 + detection (**E**–**H**). **A** and **E** all the markers reported in all studies; **B** and **F** the best markers reported in each study (avoiding considering the same sample more than once); **C**–**G** the most studied genes (*CADM1*, *FAM19A4*, *MAL*, and *miR124-2*); and **D**-**H** studies which set the threshold to achieve 70% specificity. Gray dots represent referral population-based studies, blue dots represent cohort studies, red dots represent case–control studies, and green dots represent convenience studies

Table 3 Meta-analysis of the performance of DNA methylation assays for the detection of CIN2+ and CIN3+ according to the study design

	N datasets	N samples	Pooled prevalence	Pooled sensitivity (95% CI)	Pooled specificity (95% CI)	Q (*p-value*)	I2 (95% CI)	Pooled AUC, (95% CI)
CIN2 + detection [a,b,c]								
Population Referral studies [14, 18–22, 27]	8	3627	0.28	0.60 (0.52–0.66)	0.70 (0.62–0.77)	91.489 (*p < 0.001*)	98 (96–99)	0.68 (0.64–0.72)
Cohort studies [17, 24, 28, 29, 31, 33]	6	1293	0.42	0.70 (0.62–0.76)	0.78 (0.69–0.86)	35.657 (*p < 0.001*)	94 (90–99)	0.79 (0.75–0.82)
Convenience studies [12, 13, 15, 16]	8	3234	0.20	0.69 (0.64–0.73)	0.79 (0.77–0.81)	0.478 (*p* = 0.394)	0 (0–100)	0.82 (0.78–0.85)
CIN3 + detection [a,b,d]								
Population referral studies [14, 18–23, 27]	9	4601	0.16	0.69 (0.64–0.74)	0.71 (0.64–0.78)	28.0.19 (*p < 0.001*)	93 (86–99)	0.75 (0.71–0.78)
Cohort studies [17, 24, 26, 29–31]	6	1331	0.36	0.81 (0.72–0.87)	0.72 (0.65–0.79)	27.134 (*p < 0.001*)	93 (86–99)	0.83 (0.80–0.86)
Convenience studies [12, 13, 15, 16]	8	3234	0.14	0.80 (0.75–0.84)	0.77 (0.75–0.79)	0.646 (*p* = 0.362)	0 (0–100)	0.81 (0.78–0.84)

[a] Due to the limited number of case–control studies ($n = 3$), it was not possible to perform statistical analysis. [b]The sensitivity and specificity were estimated as reported by authors. When multiple thresholds reported, 70% specificity was selected. Only one entrance per study was considered. The best combination reported by the authors was selected. [c]Only one entrance per study was considered. The best combination reported by the authors was selected. For [16] was considered *JAM3*; for [29] was considered *C13orf18/EPB41L3/JAM3*. [d]Only one entrance per study was considered. The best combination reported by the authors was selected. For H5[30] was considered PAX1/ZNF582; for K3 [29] was considered C13orf18/EPB41L3/JAM3; for J7 [27] was considered SOX1/ZSCAN1 selection bias

Table 4 Meta-analysis of the performance of DNA methylation assays for the detection of CIN2+ and CIN3+ according the sample collection method

	N Datasets	N samples	Pooled prevalence	Pooled Sensitivity, (95% CI)	Pooled Specificity, (95% CI)	Q (*p-value*)	I2 (95% CI)	Pooled AUC, (95% CI)
CIN2 + detection [a,b]								
Health professional-collected [12, 13, 16–18, 20, 24, 25, 27–29, 31–34]	18	6005	0.27	0.70 (0.65–0.74)	0.76 (0.71–0.81)	74.715 (*p < 0.001*)	97 (96–99)	0.79 (0.75–0.82)
Self-collected [14, 19, 21, 22, 28]	7	3006	0.30	0.63 (0.54–0.72)	0.73 (0.63–0.81)	84.968 (*p < 0.001*)	98 (96–99)	0.73 (0.69–0.77)
CIN3 + detection [a,c]								
Health professional-collected [12, 13, 16–18, 20, 23, 24, 26, 27, 29–32, 34]	18	6676	0.18	0.81 (0.76–0.85)	0.75 (0.70–0.79)	52.628 (*p < 0.001*)	96 (93–99)	0.85 (0.82–0.88)
Self-collected [14, 15, 19, 21, 22]	7	3006	0.19	0.72 (0.63–0.80)	0.70 (0.60–0.78)	32.355 (*p < 0.001*)	94 (88–99)	0.77 (0.74–0.81)

[a] The sensitivity and specificity were estimated as reported by authors. When multiple thresholds reported, 70% specificity was selected. [b]Only one entrance per study was considered. The best combination reported by the authors was selected. For [16] and [32] was considered *JAM3*; for [29] and [27] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier (*EPB41L3/HPV16L1/HPV16L2/HPV18L2/HPV31L1/HPV33L2*). [c]Only one entrance for study was considered. The best combination reported by the authors was selected. For [30] was considered *PAX1/ZNF582*; for [16] was considered *JAM3*; for [29] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier; for [27] was considered *SOX1/ZSCAN1*; for [32] was considered *SOX1*. [f] Only one entrance for study was considered. The best combination reported by the authors was selected. For [30] was considered *PAX1/ZNF582*; for [29] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier; for [27] was considered *SOX1/ZSCAN1*; for [32] was considered *SOX1*

cytology [using atypical squamous cells of undetermined significance (ASC-US) as cutoff] for CIN2+ detection, although with slightly lower sensitivity [42]. Concerning CIN3+ detection, methylation markers provided higher specificity with equivalent sensitivity, compared to cytology [42]. Indeed, it is widely recognized that upfront knowledge of hrHPV positivity impacts on cytological observation and reporting, usually increasing sensitivity but with a decrease in specificity [42, 43]. Except for visual inspection using acetic acid (VIA), meta-analyses of the other recommended triage strategies (genotyping and cytology) displayed lower or similar specificity than that reported for methylation markers in this meta-analysis. VIA, however, disclosed lower sensitivity for CIN3+ detection [42].

CADM1, FAM19A4, MAL, and *miR124-2* are the most commonly reported genes analyzed, although in different combinations. Six of the seven studies designed as referral population-based were conducted with these genes. However, most studies were conducted in the Dutch population, which may limit a broader application in clinical practice. Although *FAM19A4* and *miR124-2* methylation test has already received the *Conformité Européene* In Vitro *Diagnostic* (CE-IVD) label through QIAsure Methylation Test [44], concerns about its sensitivity have hindered its diffusion, especially for CIN2+ detection [13]. Additionally, although GynTect® (comprising *ASTN1, DLX1, ITGA4, RXFP3, SOX17,* and *ZNF671*) has been approved for clinical practice, it was not yet included in WHO guidelines [39].

Interestingly, Kremer and co-workers reported the association between clinical regression of high-grade CIN and a negative result in the QIAsure Methylation test [44]. In this study, women referred for colposcopy with biopsy-confirmed CIN2/CIN3 were monitored every six months during 24–30 months to evaluate clinical regression or progression of HSIL. Importantly, about 75% of women enrolled were under 35 years, i.e., at reproductive age, in which fertility preservation is of utmost importance [44]. Clinical regression was observed in 58% of recruited women, whereas clinical progression occurred in only 22%. Remarkably, a negative methylation result at baseline was associated with an increased likelihood of clinical regression. When combined with the cytological findings [ASC-US or low-grade squamous intraepithelial lesion (LSIL) or negative HPV16 genotyping ($HPV16^-$)] clinical regression incidence exceeded 85% [44]. Moreover, a report on risk stratification for $hrHPV^+$ women with ASC-US/LSIL from the same team demonstrated that a double positive test (methylation and HPV 16/18 genotyping) associated with higher risk of $CIN3^+$ incidence compared to a single positive result (methylation or HPV 16/18 genotyping). Furthermore, a double negative result associated with $CIN3^+$ incidence risk under 10% [45]. Thus, methylation analyses might identify cases more likely to endure regression. Importantly, studies with self-collected samples demonstrated a slightly lower performance. Of note, most of these studies were population-based, which might be less prone to bias design. *FAM19A4* methylation showed similar

Table 5 Pooled positive predictive value and negative predictive value according to prevalence

	N datasets	PPV (mean ± SD)	Set prevalence						
		Pooled prevalence[e]	5% PPV (mean ± SD)	10% PPV (mean ± SD)	20% PPV (mean ± SD)	30% PPV (mean ± SD)	40% PPV (mean ± SD)	50% PPV (mean ± SD)	60% PPV (mean ± SD)
CIN2 + detection									
All studies with all markers [a,b] [12–22, 24, 25, 27–29, 31–34]	56	0.620 (0.601–0.639)	0.138 (0.128–0.148)	0.252 (0.237–0.267)	0.432 (0.412–0.452)	0.565 (0.545–0.585)	0.669 (0.651–0.687)	0.752 (0.737–0.767)	0.820 (0.808–0.832)
All studies with best markers[a,c] [12–22, 24, 25, 27–29, 31–34]	25	0.514 (0.491–0.537)	0.126 (0.116–0.136)	0.233 (0.216–0.250)	0.405 (0.382–0.428)	0.538 (0.515–0.561)	0.644 (0.622–0.666)	0.731 (0.713–0.749)	0.803 (0.788–0.818)
Studies with *CADM1, FAM19A4, MAL* and/or *miR124-2*[a] [12–14, 17–22, 28, 33]	15	0.446 (0.424–0.468)	0.113 (0.104–0.122)	0.212 (0.197–0.227)	0.376 (0.355–0.397)	0.508 (0.486–0.530)	0.616 (0.595–0.637)	0.706 (0.688–0.724)	0.783 (0.768–0.798)
Set threshold to achieve 70% specificity [12, 14, 17–20, 22, 33]	9	0.478 (461–0.494)	0.106 (0.099–0.113)	0.200 (0.189–0.211)	0.359 (0.343–0.375)	0.490 (0.473.0.507)	0.599 (0.582–0.616)	0.691 (0.676–0.706)	0.770 (0.758–0.782)
CIN3 + detection									
All studies with all markers [a,b] [12–24, 26, 27, 29–32, 34]	55	0.515 (0.497–0.533)	0.151 (0.142–0.160)	0.273 (0.258–0.288)	0.457 (0.439–0.475)	0.591 (0.573–0.609)	0.692 (0.676–0.708)	0.771 (0758–0.784)	0.835 (0.825–0.845)
All studies with best markers[a,d] [12–24, 26, 27, 29–32, 34]	25	0.392 (0.371–0.413)	0.134 (0.123–0.145)	0.246 (0.229–0.263)	0.423 (0.401–0.445)	0.557 (0.513–0.579)	0.661 (0.641–0.681)	0.745 (0.734–0.756)	0.814 (0.800–0.828)
Studies with *CADM1, FAM19A4, MAL* and/or *miR124-2*[a] [12–14, 17–23]	14	0.294 (0.274–0.314)	0.119 (0.109–0.129)	0.221 (0.204–0.238)	0.389 (0.365–0.413)	0.522 (0.497–0.547)	0.629 (0.606–0.652)	0.718 (0.698–0.738)	0.792 0.776–0.808)
Set threshold to achieve 70% specificity [12, 14, 17–20, 22, 33]	9	0.347 (0.326–0.368)	0.120 (0.110–0.130)	0.224 (0.208–0.240)	0.393 (0.371–0.415)	0.526 (0.503–0.549)	0.633 (0.612–0.654)	0.721 (0.703–0.739)	0.795 (0.780–0.810)

	N studies	NPV (mean ± SD)	Set prevalence						
		Pooled prevalence[e]	5% NPV (mean ± SD)	10% NPV (mean ± SD)	20% NPV (mean ± SD)	30% NPV (mean ± SD)	40% NPV (mean ± SD)	50% NPV (mean ± SD)	60% NPV (mean ± SD)
CIN2 + detection									
All studies with all markers [a,b] [12–22, 24, 25, 27–29, 31–34]	56	0.880 (0.875–0885)	0.978 (0.977–079)	0.954 (0.952–0.956)	0.902 (0.898–0.906)	0.844 (838–850)	0.776 (0.768–0.784)	0.698 (0.688–0.708)	0.607 (0.596–0.618)

Table 5 (continued)

	N studies	NPV (mean ± SD)	Set prevalence						
		Pooled prevalence[e]	5% NPV (mean ± SD)	10% NPV (mean ± SD)	20% NPV (mean ± SD)	30% NPV (mean ± SD)	40% NPV (mean ± SD)	50% NPV (mean ± SD)	60% NPV (mean ± SD)
All studies with best markers[a,c][12–22, 24, 25, 27–29, 31–34]	25	0.857 (0.848–0.866)	0.978 (0.976–0.980)	0.954 (0.951–0.957)	0.903 (0.897–0.909)	0.844 (0.834–0.854)	0.777 (0.764–790)	0.699 (0.684–0.714)	0.608 (0.591–0.625)
Studies with *CADM1, FAM19A4, MAL* and *miR124-2*[a][12–14, 17–22, 28, 33]	15	0.858 (0.859–0.866)	0.975 (0.973–0.977)	0.948 (0.945–0.951)	0.890 (0.883–0.897)	0.825 (0.815–0.835)	0.752 (0.739–0.765)	0.669 (0.654–0.684)	0.574 (0.557–0.591)
Set threshold to achieve 70% specificity[12, 14, 17–20, 22, 33]	9	0.815 (0.804–0.826)	0.972 (0.970–0.974)	0.942 (0.938–0.946)	0.878 (0.871–0.885)	0.808 (0.797–0.819)	0.730 (0.716–0.744)	0.643 (0.627–0.659)	0.546 (0.529–0.563)
CIN3 + detection									
All studies with all markers[a,b][12–24, 26, 27, 29–32, 34]	55	0.916 (0.911–0.921)	0.985 (0.984–0.986)	0.969 (0.967–0.971)	0.933 (0.929–0.937)	0.890 (0.884–0.896)	0.839 (0.830–0.847)	0.776 (0.765–0.787)	0.698 (0.684–0.712)
All studies with best markers[a,d][12–24, 26, 27, 29–32, 34]	25	0.938 (0.932–0.006	0.984 (0.982–0.986)	0.967 (0.964–0.970)	0.929 (0.922–0.936)	0.885 (0.875–0-895)	0.832 (0.818–8.46)	0.768 (0.750–0.783)	0.688 (0.666–0.710)
Studies with *CADM1, FAM19A4, MAL* and *miR124-2*[a][12–14, 17–23]	14	0.939 (0.934–0.944)	0.979 (0.977–0.981)	0.958 (0.954–0.962)	0.910 (0.903–0.917)	0.855 (0.844–0.866)	0.791 (0.777–0.805)	0.716 (0.698–0.734)	0.627 (0.607–0.647)
Set threshold to achieve 70% specificity[12, 14, 17–20, 22, 33]	9	0.919 (0.912–0.926)	0.978 (0.976–0.980)	0.955 (0.951–0.959)	0.903 (0.895–0.911)	0.845 (0.833–0.857)	0.778 (0.762–0.794)	0.701 (0.681–0.721)	0.610 (0.588–0.632)

[a] The sensitivity and specificity were estimated as reported by authors. When multiple thresholds reported, 70% specificity was selected. [b]Pooled together all the genes and gene combination reported in each study. For studies [16, 23, 25, 26, 29, 32], more than one entrance was considered. [c]Only one entrance per study was considered. The best combination reported by the authors was selected. For [16] and [32] was considered *JAM3*; for [29] and [27] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier (*EPB41L3/HPV16L1/HPV16L2/HPV18L2/HPV31L1/HPV33L2*). [d]Only one entrance for study was considered. The best combination reported by the authors was selected. For [30] was considered *PAX1/ZNF582*; for [16] was considered *JAM3*; for [29] was considered *C13orf18/EPB41L3/JAM3*; for [25] was considered S5 classifier; for [27] was considered *SOX1/ZSCAN1*; for [32] was considered *SOX1*. [e]Presented in Table 2

performance in self-collected and heath professional-collected samples [18, 19]. Moreover, the study of Kremer et al. also demonstrated similar test performance for clinician- and self-collected samples, encouraging the adoption of this strategy for recruitment of women non-adherent to screening programs, increasing screening uptake as proposed by HPV Action Network 2 and allowing for fully automated molecular testing pipeline.

Furthermore, Kelly et al. [46] also demonstrated the value of methylation markers for cancer detection (not restricted to hrHPV-positive women). Overall, methylation markers disclosed 63% and 71% sensitivity and 76% and 75% specificity, for CIN2+ and CIN3+, respectively [46]. These results emphasize the benefit of using methylation markers for cervical cancer screening.

Conclusions

In conclusion, this systematic review and meta-analysis confirmed that DNA methylation-based markers constitute a promising tool for hrHPV-positive women in cervical cancer screening programs as its higher specificity complements the high sensitivity of hrHPV testing. In addition to decreased overdiagnosis and consequent overtreatment, increasing quality of life and reducing healthcare costs, this strategy may also contribute to decrease pressure upon colposcopy units, improving sustainability and waiting times. Cervical cancer screening program evolution over the decades has been constant, in search of the optimal balance between effectiveness and reliability. Methylation markers may well be the next advancement, improving adhesion, cost-effectiveness, and quality of life.

Quality assessment

Two authors (SS and JL) assessed the quality of studies using the QUADAS-2 [54] tool. Discrepancies were solved by a third author (BM). Bias was assessed based on: participant selection (population characteristics, inclusion and exclusion criteria, and proportion of women with CIN2+/CIN3+ included), index test description (DNA methylation assessment description and cutoff for methylation positivity), and reference test (histological confirmation assessment). Additional file 1: Table S2 depicts the quality assessment for all the included studies.

This analysis is reported according to the Preferred Reporting Items of Systematic Reviews and Meta-analysis of Diagnostic Test Accuracy Studies (PRISMA-DTA) guidelines [55, 56] and the present review was registered on the PROSPERO database at the Centre of Reviews and Dissemination, University of York, UK, with the registration number CRD42022350086 (https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=350086).

Abbreviations

ANKRD18CP	Ankyrin repeat domain 18C, pseudogene
ASCL1	Achaete-scute family bHLH transcription factor 1
ASC-US	Atypical squamous cells of undetermined significance
AUC	Area under the curve
CADM1	Cell adhesion molecule 1
CE-IVD	Conformité Européene in vitro diagnostic
CIN	Cervical intraepithelial neoplasia
DLX 1	Distal-less homeobox 1
EPB41L3	Erythrocyte membrane protein band 4.1 like 3
FN	False negatives
FP	False positives
hrHPV	High-risk human papillomavirus
HSIL	High-grade intraepithelial lesions
ICC	Invasive cervical carcinoma
ITGA4	Integrin subunit alpha 4
JAM3	Junctional adhesion molecule 3
LHX8	LIM homeobox 8
MAL	Mal, T cell differentiation protein
miR124-2	MicroRNA 124-2
NPV	Negative predictive values
PAX1	Paired box 1
PPV	Positive predictive values
ROC	Receiver operating characteristics
RUBCNL	Rubicon-like autophagy enhancer
RXFP3	Relaxin family peptide receptor 3
SLIT2	Slit guidance ligand 2
SOX 17	SRY-box transcription factor 17
SOX1	SRY-box transcription factor 1
SROC	Summary receiver operating characteristics
ST6GALNAC5	ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5
TAFA4	TAFA chemokine-life family member 4
TERT	Telomerase reverse transcriptase
TN	True negatives
TP	True positives
ZCAN1	Zinc finger and SCAN domain containing 1
ZNF582	Zinc finger protein 582
ZNF671	Zinc finger protein 671

Supplementary Information

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Additional file 1. Additional Tables S1–S2.

Additional file 2. Additional Figures S1–S4 and Additional Methods.

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Author contributions

SS and JL searched the literature, selected and extracted the data for statistical analysis, and drafted the manuscript. BM played as third reader for discrepancies in data selection and extraction. SS and BM performed the statistical analysis. RH and CJ critically revised the manuscript and supervised the work. All authors read and approved the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Cancer Biology & Epigenetics Group, Research Center of IPO Porto (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal. [2]Doctoral Program in Molecular Pathology and Genetics, School of Medicine & Biomedical Sciences (ICBAS-UP), Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. [3]ESS, Polytechnic of Porto, Rua Dr. António Bernardino de Almeida, 400, 4200-072 Porto, Portugal. [4]Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal. [5]Department of Pathology and Molecular Immunology, School of Medicine and Biomedical Sciences (ICBAS-UP), Rua Jorge Viterbo Ferreira 228, 4050-513 Porto, Portugal. [6]Oncology Nursing Research Unit, Research Center of IPO Porto (CI-IPOP) /CI-IPOP@RISE (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal. [7]School of Health, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal. [8]Clinical Academic Centre of Trás-os-Montes and Alto Douro (CACTMAD), Vila Real, Portugal.

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Additional file 1

Additional Table S1

Table S1 - Collected variables for each study

ID	Population overlap	Number controls/ CIN0	Number CIN1	Number CIN2	Number CIN3	Number IC	Cytology TP for CIN2+ cases (ASC-US)	Cytology TN for CIN2+ cases (ASC-US)	Cytology FP for CIN2+ cases (ASC-US)	Cytology FN for CIN2+ cases (ASC-US)	HPV 16/18 test TP for CIN2+	HPV 16/18 test FP for CIN2+	HPV 16/18 test TN for CIN2+	HPV 16/18 test FN for CIN2+	DNA methylation markers studied	Cutoff	Method for cutoff determination - ROC (other)	Number CIN2+ positives (TP)	Number sCIN1 negatives (TN)	Number sCIN1 positives (FP)	Number CIN2+ negatives (FN)	Number of CIN2+	Number of sCIN1 positives	Number CIN3+ positives (TP)	Number sCIN2 negatives (TN)	Number sCIN2 positives (FP)	Number CIN3+ negatives (FN)	Number of CIN3+	Number of sCIN2 positives	Ref.
B1	No	178[a]		20	35	4	38	149	29	20	---	---	---	---	CADM1/MAL	Positive of at least 1 marker	ROC spec. ≥70%	55	42	136	3			38	45	153	0			Hesselink et al. 2011 Clin Can Res
																	ROC spec. ≥80%	52	59	119	6			37	64	134	1			
																	ROC spec. ≥90%	46	79	99	12			33	86	112	5			
																	ROC spec. ≥95%	44	96	82	14	58	178	32	104	94	6	38	198	
																	ROC spec. ≥98%	42	115	63	16			31	124	74	7			
																	ROC spec. ≥70%	36	139	39	22			26	149	49	12			
																	ROC spec. ≥80%	30	153	25	28			23	165	33	15			
E1	Yes	58	21	15	25	9	41	40	8	39	---	---	---	---	C13ORF18 JAM3 EPB41L3 TERT	ROC Curve	ROC spec. ≥70%	24	73	6	25	79	166	22	86	8	12	34	94	Boers et al. 2014 BJC
																		31	71	8	18			28	83	11	6			
																		33	45	37	16	49	79	30	57	37	4	34	94	
E4	No	8+147[b]	11	19	30	3	33	139	27	19	---	---	---	---	FAM19A4	ROC Curve	ROC spec. ≥70%	36	116	50	16	52	166	26	56	38	8	33	185	De Strooper et al. 2014 Can Prevent Res
E5	No	105	28	42	23	19	---	---	---	---	---	---	---	---	DLX1/ITGA4/ RXFP3/SOX17/ZNF671	Positive of at least 2 of 5 markers positive	Convience	47	118	15	37	84	133	32	145	30	10	42	175	Hesselink et al. 2014 Hist Cyto
E9	Yes	231+165+ 247[c]	151	78	134	13	---	---	---	---	---	133	267	527	MAL/miR124-2	ROC curve	ROC spec. ≥50%	163	389	405	62	225	794	108	412	460	39	147	872	Verhoef et al. 2014 Gynecologic Oncology
																	ROC spec. ≥60%	150	454	340	75			100	482	390	47			
																	ROC spec. ≥70%	111	541	253	114			81	589	283	66			
																	ROC spec. ≥80%	76	650	155	149			66	724	148	81			
E10	Yes	133+72+44[d]	79	45	73	6	---	---	---	---	---	---	---	---	MAL/miR124-2	Positive of at least 1 marker	ROC spec. ≥50%	90	121	163	34	124	284	55	131	198	24	79	329	Verhoef et al. 2014 Lancet Onc
																	ROC spec. ≥60%	83	16	139	0			---	---	---	---	---	---	
																	ROC spec. ≥70%	80	53	102	1			---	---	---	---	---	---	
																	ROC spec. ≥80%	67	60	95	6			---	---	---	---	---	---	
F1	No	71	84	50	43	0	88	71	84	5	41	37	118	52	CADM1/MAL/miR124-2	Positive of at least 1 marker	ROC spec. ≥90%	83	78	77	10	93	155	---	---	---	---	---	---	Vuyst et al. 2014 Epidemiology and Prevention
																	ROC spec. ≥95%	79	91	64	14			---	---	---	---	---	---	
																	ROC spec. ≥98%	67	109	46	26			---	---	---	---	---	---	
																	ROC spec. ≥70%	60	124	31	33			---	---	---	---	---	---	
																	ROC spec. ≥80%	47	140	15	46			---	---	---	---	---	---	
F5	Yes	77+40+93[e]	64	28	56	6	68	215	59	22	---	---	---	---	CADM1/MAL	Positive of at least 1 marker	ROC spec. ≥70%	54	198	76	36	90	274	43	215	87	19	62	302	Verhoef et al. 2015 Gynecologic Oncology
F6	No	55	41	41	24	7	---	---	---	---	---	---	---	---	JAM3	ROC Curve	ROC Curve	42	86	10	30	72	96	25	110	27	6	31	137	Yin et al. 2015 Oncotarget
G1[f]	Yes	125+69[g]	76	41	72	6	---	---	---	---	---	67	93	177	52	FAM19A4/miR124-2	Positive of at least 1 marker	ROC spec. ≥70%	76	191	79	43	119	270	55	211	100	23	78	311	De Strooper et al. 2016 Gynecologic Oncology
G1[h]		24+107[g]	24	27	68	5	---	---	---	---	---	64	39	116	35			ROC spec. ≥70%	99	121	34	40	99	155	50	139	43	22	72	182	
G2	Yes	41+146+89[c]	26	20	19[+]		---	---	---	21	89	213	18	5S (EPB41L3 + HPV16L1 + HPV16L2+ HPV18L2 + HPV31L1 +HPV33L2) 54 (EPB41L3 + HPV16L1 + HPV16L2 + HPV18L2 + HPV31L1)	0.8 0.5	Sens. >90%	29	197	105	10	39	302	---	---	---	---	---	---	Lorincz et al. 2016 IJC
																		27	178	124	12	39	302	---	---	---	---	---	---	
G3	Yes	173	124	78	73	2	---	---	---	---	---	---	---	---	FAM19A4	ROC Curve	ROC spec. ≥70%	68	246	51	85	153	297	49	305	70	26	75	375	Luttmer et al. 2016 BJC
G4	Yes	190	138	90	87	3	---	---	---	---	---	---	---	---	FAM19A4	0.415	Unclear	104	243	85	76	180	328	68	297	121	22	90	418	Luttmer et al. 2016 IJC
H4	No	58	42[k]		84	5	---	---	---	---	---	---	---	---	ASTN1/DLX1/ITGA4/RXFP3/SOX17/ZNF671	Added score of all six genes higher or equal to 0.5		---	---	---	---	---	---	---	---	---	---	89	100	Schmitz et al. 2017 Clin Epigen
H5	No	100	18	39	86	69	---	---	---	115	24	94	79	ZNF582	AUC ≥11	Positive of at least 1 marker	Under	---	---	---	---	---	---	60	76	24	29	155	157	Tian et al. 2017 Oncotarget
															ZNF582/PAX1	ROC ≥11		---	---	---	---	---	---	120	122	35	35			
H7	No	45	50	46	43	75	---	---	---	---	---	---	---	---	JAM3	Unclear	Under	146	86	9	18	164	95	134	102	55	21	118	141	Yuan et al. 2017 Int J Clin Exp Med
															SLIT2			100	85	10	64			98	127	14	20			
															TERT			150	43	52	64			83	111	30	35			
															SOX1			106	94	2	108			101	56	85	17			
															C13orf18			120	94	1	44			101	123	18	17			
															JAM3/SLIT2			125	87	8	39			87	128	13	31			
															JAM3/C13orf18			128	67	28	36			89	106	35	29			
															JAM3/SOX1			118	66	30	48			103	117	24	15			
															SLIT2/C13orf18			116	65	34	48			78	109	32	40			
															SLIT2/SOX1			126	85	10	38			90	128	13	28			
															C13orf18/SOX1			98	78	17	66			79	127	14	39			
I1	No	66	31	5[+]		61	98	58	39	20	77	32	65	41	FAM19A4	Under	Under	89	79	18	29	118	97	---	---	---	---	---	---	Bu et al. 2018 BMC Cancer
I4[i]	Yes	178[k]		22	35	8	---	---	---	---	---	---	---	---	ASCL1/UHX0/	Logistic Model	80% specificity	45	106	28	20	65	134	34	117	39	9	43	156	Verlaat et al. 2018 Clin Cancer Res
a[j]		134[k]		28	56	25	---	---	---	---	---	---	---	---	ST6GALNAC5			88	144	34	21	109	178	74	158	48	7	81	206	
J1	No	150[l]		63	44	0	---	---	---	---	---	---	---	---	5S (EPB41L3 + HPV16L1 + HPV16L2+ HPV18L2 + HPV31L1 +HPV33L2)	0.8	Logistic Model	81	66	84	26	107	150	41	89	124	3	44	213	Cook et al. 2019 IJC
J7	No	189	11	16	16	3	---	---	---	---	---	---	---	---	ANKRD18CP C13ORF18 EPB41L3 SOX1 ZSCAN1 ANKRD18CP/ C13ORF18/EPB41L3 C13orf18/EPB41L3/ JAM3 SOX1/ZCAN1	Ct below 50 Ct below 50 Ct below 50 Methylation above 19.1 Methylation above 132 Positive of at least 1 marker	Under	16 8 25 17 21 15 21 28 22	144 101 137 108 173 176 136 132 107	56 9 63 12 27 24 64 68 33	19 27 10 14 20 14 7 13	35	200	9 6 16 14 21 11 13 18 15	153 205 205 182 188 144 127 176	63 11 73 16 34 28 72 89 40	10 13 3 6 5 8 6 1 4	19	216	van Leeuwen et al. 2019 IJC
K3	No	66	32	46	49	34	---	---	---	---	---	---	---	---	C13orf18/JAM3 ANKRD18CP/ C13orf18/JAM3 SOX1/ZCAN1 ANKRD18CP C13ORF18 EPB41L3 JAM3 SOX1	Ct below 50 Ct below 50 Ct below 50 Ct below 50 Ct below 50 Methylation above 19.1	Unclear	102 97 92 53 94 87 87	62 66 66 98 66 74 80	36 32 32 0 32 24 18	27 32 37 71 35 42 42	129	98	77 75 74 68 47 74 72 73	84 91 94 103 140 92 107 112	60 53 50 36 4 52 37 32	6 8 9 15 36 9 11 10	83	144	Li et al. 2020 IJC

															ZSCAN1	Methylation above 132		84	76	22	45			69	107	37	14			
L1[a]	No	80+23[b]	18	15	20	5	---	---	---	---	---	---	---	---	FAM19A4/miR124-2	Manufacturer	ROC Curve	25	86	35	15	40	121	20	96	40	5	25	136	Bonde et al. 2021 IJC
L1[b]		193+122[b]	34	18	49	8												55	277	72	20	75	349	44	284	83	13	57	367	
L1[c]		59+692[b]	75	42	58	2												69	637	189	33	102	826	47	657	211	13	60	868	
L1[d]		32+648[b]	36	49	101	5												104	575	141	51	155	716	84	604	161	22	106	765	
L2	Yes	864			109	6	---	---	---	---	---	---	---	---	FAM19A4/miR124-2	Positive of at least 1 marker	ROC Curve	----	---	---	----	----	---	87	675	189	28	115	864	Vink et al. 2021 Clin Micr Inf

*a Included histologically confirmed CIN0 or CIN1, or negative co-test (cytology and hrHPV) at follow-up; *b histologically confirmed CIN0 + negative co-test (cytology and hrHPV) at follow-up; *c histologically confirmed CIN0 + negative co-test (cytology and hrHPV) at follow-up+ women without study endpoint (classified by the authors as 'no CIN2+ detected'); *d women without study endpoint were excluded since was not clear how many were classified as control and cases; *e reported as a group; *f Included histologically confirmed CIN0 or CIN1, or negative co-test (cytology and hrHPV) at follow-up and women without exit endpoint. *---* - not available/applicable; **Abbreviations:** ASC-US: Atypical Squamous Cell of Undeterminated Significance; CIN: Cervical Intraephitelail Neoplasia; Ct: Cycle Threshold; FN: False Negatives; FP: False Positives; IC: Invasive Cancer; ROC: Receiver Operating Characteristic; TN: True Negatives; TP: True Positives.

Table S2 – Individual studies quality assessment according to QUADAS-2.

Author, year, journal	StudyID	Risk of bias																	Concerns of applicability		
		Patient selection					Index test				Reference standard				Flow and timing				Patient selection	Index test	Reference standard
		P1	P2	P3	P4	P	T1	T2	T3	T	R1	R2	R3	R	F1	F2	F3	F			
Referral population based Studies																					
De Strooper et al. 2016 Gyn Onc	G1	Y	Y	N	Y	Possible	Y	U	Y	Low	Y	Y	U	Low	Y	Y	N*	Low*	Possible	Low	Low*
Luttmer et al. 2016 BJC	G3	Y	Y	N	Y	Possible	Y	U	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Possible	Low	Low
Luttmer et al. 2016 IJC	G4	Y	Y	N	Y	Possible	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Possible	Low	Low
van Leeuwen et al. 2019 IJC	J7	Y	Y	Y	Y	Low	Y	Y	Y	Low	U	U	Y	Possible	N	U	U	Possible	Low	Low	Possible
Verhoef et al. 2014 Gynecologic Oncology	E9	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Y	U	N*	Low*	Low	Low	Low*
Verhoef et al. 2014 Lancet Oncol	E10	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	N*	Low*	Low	Low	Low*
Verhoef et al. 2015 Gyn Onc	F5	Y	Y	Y	Y	Low	Y	U	Y	Low	Y	Y	Y	Low	Y	Y	N	Low*	Low	Low	Low
Cohort Studies																					
Bu et al. 2018 BMC Cancer	I1	U	Y	N	U	High	Y	Y	Y	Low	U	U	U	Possible	Y	U	N	Possible	High	Low	Possible
De Strooper et al. 2014 Canc Prev Res	E4	Y	U	Y	U	Possible	Y	U	Y	Low	Y	Y	Y	Low	N	Y	N*	Low*	Possible	Low	Low*
Hansel et al. 2014 Plos One	E5	Y	Y	N	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	N	U	Y	Possible	Possible	Low	Low
Li et al. 2020 IJC	K3	Y	Y	N	Y	Possible	Y	Y	Y	Low	Y	Y	U	Low	N	U	Y	High	Possible	Low	Low
Schmitz et al. 2017 Clin Epigen	H4	U	U	N	N	High	Y	U	Y	Low	Y	Y	Y	Low	N	Y	Y	Possible	High	Low	Low
Tain et al. 2017 Oncotarget	H5	Y	Y	N	Y	Possible	Y	U	Y	Low	Y	Y	Y	Low	N	U	Y	Possible	Possible	Low	Low
Vuyst et al. 2014 Epidemiology and Prevention	F1	Y	Y	N	Y	Possible	Y	U	Y	Low	Y	Y	U	Low	Y	U	Y	Low	High	Low	Low
Yin et al. 2015 Oncotarget	F6	U	Y	N	U	High	Y	Y	Y	Low	Y	Y	Y	Low	N	U	Y	Possible	High	Low	Low
Case-control Studies																					
Cook et al. 2019 IJC	J1	Y	U	N	U	High	Y	Y	Y	Low	Y	Y	Y	Low	N	Y	N	High	High	Low	Low
Lorincz et al. 2016 IJC	G2	Y	U	Y	U	Possible	Y	Y	Y	Low	U	U	Y	Possible	N	U	U	High	Possible	Low	Possible
Yuan et al. 2017 Int J Clin Exp	H7	U	U	N	Y	High	Y	U	U	Possible	Y	Y	Y	Low	N	Y	Y	Low	High	Possible	Low
Convenience studies																					
Bonde et al. 2021 IJC	L1	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	N	Y	N*	Low*	Low	Low	Low*
Boer et al. 2014 BJC	E1	Y	U	N	U	High	Y	U	Y	Low	Y	Y	Y	Low	N	U	N	High	High	Low	Possible
Hessenlink et al. 2011 Clin Canc Res	B1	Y	Y	Y	U	Low	Y	U	Y	Low	N	Y	Y	Possible	N	U	N	High	Low	Low	Possible
Verlaat et al. 2018 Clin Cancer Res	I8	Y	Y	N	Y	Low	Y	U	Y	Low	Y	Y	Y	Low	N	U	N*	Possible	Low	Low	Low*
Vink et al. 2021 Clin Micro Inf	L2	Y	Y	Y	Y	Low	Y	U	Y	Low	U	Y	Y	Possible	Y	Y	U	Possible	Low	Low	Possible

QUADAS items: P1=Participant selection is fully described; P2= A consecutive or random sample of patients was selected P3= Study population had a prevalence of CIN2+ below 30% (in studies reporting only CIN3+ outcome prevalence <20%); P4= Study avoided innappropiate exclusions; T1= The index test was well described; T2= The index test results were intrepeted without knowlegde of the results of reference standard; T3=The treshold used was pre-specified; R1= The reference test was well described; R2= The refence standard was likely to correctly the target; R3= The reference standard results were interpreted without knowlegde of the results of index test; F1= Description of women lost-to-follow up; F2=Appropriate interval between index and reference test; F3= All the patients received the same reference standard (i.e all the women had histological result); Y=fulfilled; N=not fulfilled; U=Unclear *co-test after 6 months with a double-negative result have a low risk of misclassification

Additional file 2

Additional Figures Legends

Additional Figure S1 – Forest plot of individual studies for sensitivity (left panel) and specificity (right panel) for CIN2+ detection considering the best marker reported in each study. Top studies (gray): referral population-based studies; Middle upper (blue): cohort studies; Middle bottom (red): case-control studies; Bottom (green): convenience studies.

Additional Figure S2 – Forest plot of individual studies for sensitivity (left panel) and specificity (right panel) for CIN3+ detection considering the best marker reported in each study. Top studies (gray): referral population-based studies; Middle upper (blue): cohort studies; Middle bottom (red): case-control studies; Bottom (green): convenience studies.

Additional Figure S3 – Univariable meta-regression for CIN 2+ (A-D) and CIN3+ detection (E-H). (A and E) all the markers reported in all studies; (B and F) the best markers reported in each study (avoiding considering the same sample more than once); (C-G) the most studied genes (*CADM1, FAM19A4, MAL,* and *miR124-2*); and (D-H) studies which set the threshold to achieve 70% specificity.

Additional Figure S4 – Positive predictive value (PPV) in red and Negative predictive value (NPV) in blue for CIN 2+ (A-D) and CIN3+ detection (E-H). (A and E) all the markers reported in all studies; (B and F) the best markers reported in each study (avoiding considering the same sample more than once); (C-G) the most studied genes (*CADM1, FAM19A4, MAL,* and *miR124-2*); and (D-H) studies which set the threshold to achieve 70% specificity.

Figure S1

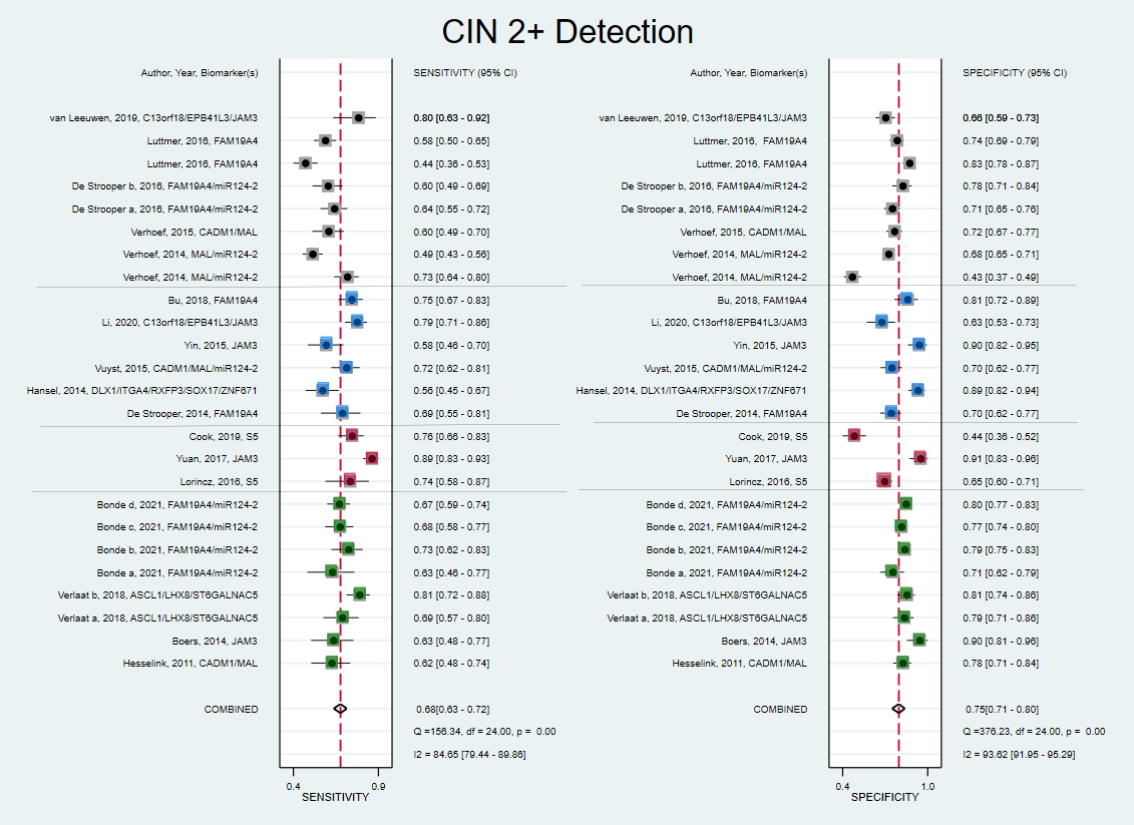

CIN 2+ Detection
Author, Year, Biomarker(s)
SENSITIVITY (95% CI)
van Leeuwen, 2019, C13orf18/EPB41L3/JAM3 0.80 [0.63 - 0.92]
Luttmer, 2016, FAM19A4 0.58 [0.50 - 0.65]
Luttmer, 2016, FAM19A4 0.44 [0.36 - 0.53]
De Strooper b, 2016, FAM19A4/miR124-2 0.60 [0.49 - 0.69]
De Strooper a, 2016, FAM19A4/miR124-2 0.64 [0.55 - 0.72]
Verhoef, 2015, CADM1/MAL 0.60 [0.49 - 0.70]
Verhoef, 2014, MAL/miR124-2 0.49 [0.43 - 0.56]
Verhoef, 2014, MAL/miR124-2 0.73 [0.64 - 0.80]
Bu, 2018, FAM19A4 0.75 [0.67 - 0.83]
Li, 2020, C13orf18/EPB41L3/JAM3 0.79 [0.71 - 0.86]
Yin, 2015, JAM3 0.58 [0.46 - 0.70]
Vuyst, 2015, CADM1/MAL/miR124-2 0.72 [0.62 - 0.81]
Hansel, 2014, DLX1/ITGA4/RXFP3/SOX17/ZNF671 0.56 [0.45 - 0.67]
De Strooper, 2014, FAM19A4 0.69 [0.55 - 0.81]
Cook, 2019, S5 0.76 [0.66 - 0.83]
Yuan, 2017, JAM3 0.89 [0.83 - 0.93]
Lorincz, 2016, S5 0.74 [0.58 - 0.87]
Bonde d, 2021, FAM19A4/miR124-2 0.67 [0.59 - 0.74]
Bonde c, 2021, FAM19A4/miR124-2 0.68 [0.58 - 0.77]
Bonde b, 2021, FAM19A4/miR124-2 0.73 [0.62 - 0.83]
Bonde a, 2021, FAM19A4/miR124-2 0.63 [0.46 - 0.77]
Verlaat b, 2018, ASCL1/LHX8/ST6GALNAC5 0.81 [0.72 - 0.88]
Verlaat a, 2018, ASCL1/LHX8/ST6GALNAC5 0.69 [0.57 - 0.80]
Boers, 2014, JAM3 0.63 [0.48 - 0.77]
Hesselink, 2011, CADM1/MAL 0.62 [0.48 - 0.74]
COMBINED 0.68[0.63 - 0.72]
Q =156.34, df = 24.00, p = 0.00
I2 = 84.65 [79.44 - 89.86]
0.4
0.9
SENSITIVITY
Author, Year, Biomarker(s)
SPECIFICITY (95% CI)
van Leeuwen, 2019, C13orf18/EPB41L3/JAM3 0.66 [0.59 - 0.73]
Luttmer, 2016, FAM19A4 0.74 [0.69 - 0.79]
Luttmer, 2016, FAM19A4 0.83 [0.78 - 0.87]
De Strooper b, 2016, FAM19A4/miR124-2 0.78 [0.71 - 0.84]
De Strooper a, 2016, FAM19A4/miR124-2 0.71 [0.65 - 0.76]
Verhoef, 2015, CADM1/MAL 0.72 [0.67 - 0.77]
Verhoef, 2014, MAL/miR124-2 0.68 [0.65 - 0.71]
Verhoef, 2014, MAL/miR124-2 0.43 [0.37 - 0.49]
Bu, 2018, FAM19A4 0.81 [0.72 - 0.89]
Li, 2020, C13orf18/EPB41L3/JAM3 0.63 [0.53 - 0.73]
Yin, 2015, JAM3 0.90 [0.82 - 0.95]
Vuyst, 2015, CADM1/MAL/miR124-2 0.70 [0.62 - 0.77]
Hansel, 2014, DLX1/ITGA4/RXFP3/SOX17/ZNF671 0.89 [0.82 - 0.94]
De Strooper, 2014, FAM19A4 0.70 [0.62 - 0.77]
Cook, 2019, S5 0.44 [0.36 - 0.52]
Yuan, 2017, JAM3 0.91 [0.83 - 0.96]
Lorincz, 2016, S5 0.65 [0.60 - 0.71]
Bonde d, 2021, FAM19A4/miR124-2 0.80 [0.77 - 0.83]
Bonde c, 2021, FAM19A4/miR124-2 0.77 [0.74 - 0.80]
Bonde b, 2021, FAM19A4/miR124-2 0.79 [0.75 - 0.83]
Bonde a, 2021, FAM19A4/miR124-2 0.71 [0.62 - 0.79]
Verlaat b, 2018, ASCL1/LHX8/ST6GALNAC5 0.81 [0.74 - 0.86]
Verlaat a, 2018, ASCL1/LHX8/ST6GALNAC5 0.79 [0.71 - 0.86]
Boers, 2014, JAM3 0.90 [0.81 - 0.96]
Hesselink, 2011, CADM1/MAL 0.78 [0.71 - 0.84]
COMBINED 0.75[0.71 - 0.80]
Q =376.23, df = 24.00, p = 0.00
I2 = 93.62 [91.95 - 95.29]
0.4
1.0
SPECIFICITY

Figure S2

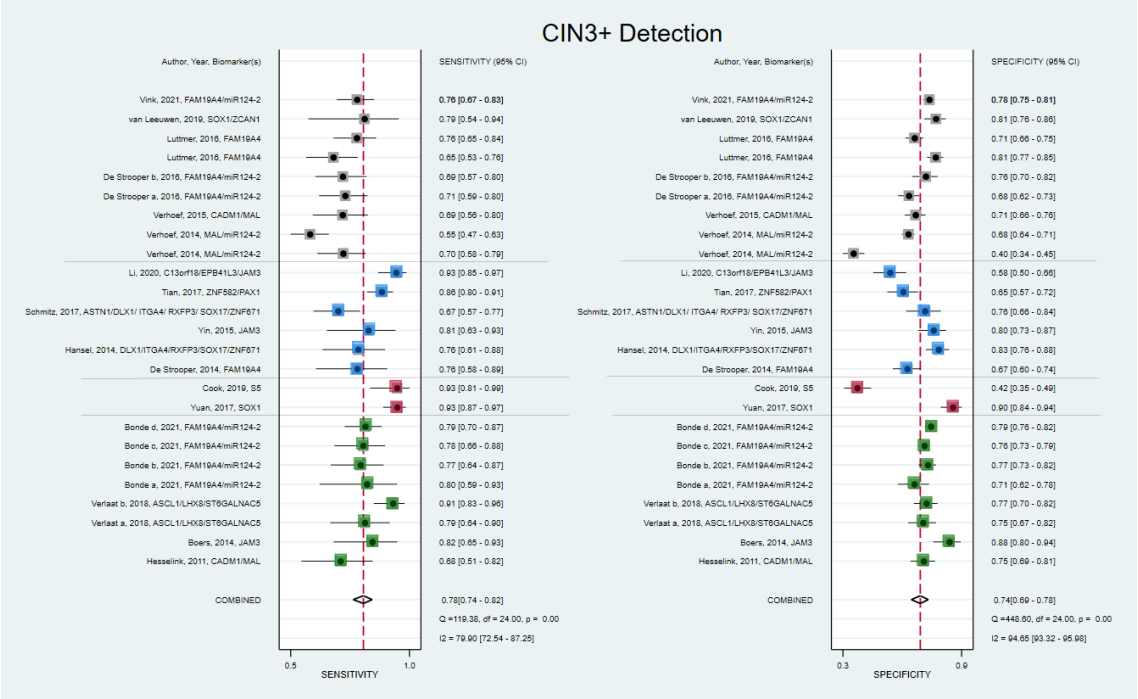

CIN3+ Detection
Author, Year, Biomarker(s)
SENSITIVITY (95% CI)
Vink, 2021, FAM19A4/miR124-2 0.76 [0.67 - 0.83]
van Leeuwen, 2019, SOX1/ZCAN1 0.79 [0.54 - 0.94]
Luttmer, 2016, FAM19A4 0.76 [0.65 - 0.84]
Luttmer, 2016, FAM19A4 0.65 [0.53 - 0.76]
De Strooper b, 2016, FAM19A4/miR124-2 0.69 [0.57 - 0.80]
De Strooper a, 2016, FAM19A4/miR124-2 0.71 [0.59 - 0.80]
Verhoef, 2015, CADM1/MAL 0.69 [0.56 - 0.80]
Verhoef, 2014, MAL/miR124-2 0.55 [0.47 - 0.63]
Verhoef, 2014, MAL/miR124-2 0.70 [0.58 - 0.79]
Li, 2020, C13orf18/EPB41L3/JAM3 0.93 [0.85 - 0.97]
Tian, 2017, ZNF582/PAX1 0.86 [0.80 - 0.91]
Schmitz, 2017, ASTN1/DLX1/ ITGA4/ RXFP3/ SOX17/ZNF671 0.67 [0.57 - 0.77]
Yin, 2015, JAM3 0.81 [0.63 - 0.93]
Hansel, 2014, DLX1/ITGA4/RXFP3/SOX17/ZNF671 0.76 [0.61 - 0.88]
De Strooper, 2014, FAM19A4 0.76 [0.58 - 0.89]
Cook, 2019, S5 0.93 [0.81 - 0.99]
Yuan, 2017, SOX1 0.93 [0.87 - 0.97]
Bonde d, 2021, FAM19A4/miR124-2 0.79 [0.70 - 0.87]
Bonde c, 2021, FAM19A4/miR124-2 0.78 [0.66 - 0.88]
Bonde b, 2021, FAM19A4/miR124-2 0.77 [0.64 - 0.87]
Bonde a, 2021, FAM19A4/miR124-2 0.80 [0.59 - 0.93]
Verlaat b, 2018, ASCL1/LHX8/ST6GALNAC5 0.91 [0.83 - 0.96]
Verlaat a, 2018, ASCL1/LHX8/ST6GALNAC5 0.79 [0.64 - 0.90]
Boers, 2014, JAM3 0.82 [0.65 - 0.93]
Hesselink, 2011, CADM1/MAL 0.68 [0.51 - 0.82]
COMBINED 0.78[0.74 - 0.82]
Q =119.38, df = 24.00, p = 0.00
I2 = 79.90 [72.54 - 87.25]
0.5
1.0
SENSITIVITY
Author, Year, Biomarker(s)
SPECIFICITY (95% CI)
Vink, 2021, FAM19A4/miR124-2 0.78 [0.75 - 0.81]
van Leeuwen, 2019, SOX1/ZCAN1 0.81 [0.76 - 0.86]
Luttmer, 2016, FAM19A4 0.71 [0.66 - 0.75]
Luttmer, 2016, FAM19A4 0.81 [0.77 - 0.85]
De Strooper b, 2016, FAM19A4/miR124-2 0.76 [0.70 - 0.82]
De Strooper a, 2016, FAM19A4/miR124-2 0.68 [0.62 - 0.73]
Verhoef, 2015, CADM1/MAL 0.71 [0.66 - 0.76]
Verhoef, 2014, MAL/miR124-2 0.68 [0.64 - 0.71]
Verhoef, 2014, MAL/miR124-2 0.40 [0.34 - 0.45]
Li, 2020, C13orf18/EPB41L3/JAM3 0.58 [0.50 - 0.66]
Tian, 2017, ZNF582/PAX1 0.65 [0.57 - 0.72]
Schmitz, 2017, ASTN1/DLX1/ ITGA4/ RXFP3/ SOX17/ZNF671 0.76 [0.66 - 0.84]
Yin, 2015, JAM3 0.80 [0.73 - 0.87]
Hansel, 2014, DLX1/ITGA4/RXFP3/SOX17/ZNF671 0.83 [0.76 - 0.88]
De Strooper, 2014, FAM19A4 0.67 [0.60 - 0.74]
Cook, 2019, S5 0.42 [0.35 - 0.49]
Yuan, 2017, SOX1 0.90 [0.84 - 0.94]
Bonde d, 2021, FAM19A4/miR124-2 0.79 [0.76 - 0.82]
Bonde c, 2021, FAM19A4/miR124-2 0.76 [0.73 - 0.79]
Bonde b, 2021, FAM19A4/miR124-2 0.77 [0.73 - 0.82]
Bonde a, 2021, FAM19A4/miR124-2 0.71 [0.62 - 0.78]
Verlaat b, 2018, ASCL1/LHX8/ST6GALNAC5 0.77 [0.70 - 0.82]
Verlaat a, 2018, ASCL1/LHX8/ST6GALNAC5 0.75 [0.67 - 0.82]
Boers, 2014, JAM3 0.88 [0.80 - 0.94]
Hesselink, 2011, CADM1/MAL 0.75 [0.69 - 0.81]
COMBINED 0.74[0.69 - 0.78]
Q =448.60, df = 24.00, p = 0.00
I2 = 94.65 [93.32 - 95.98]
0.3
0.9
SPECIFICITY

Figure S3

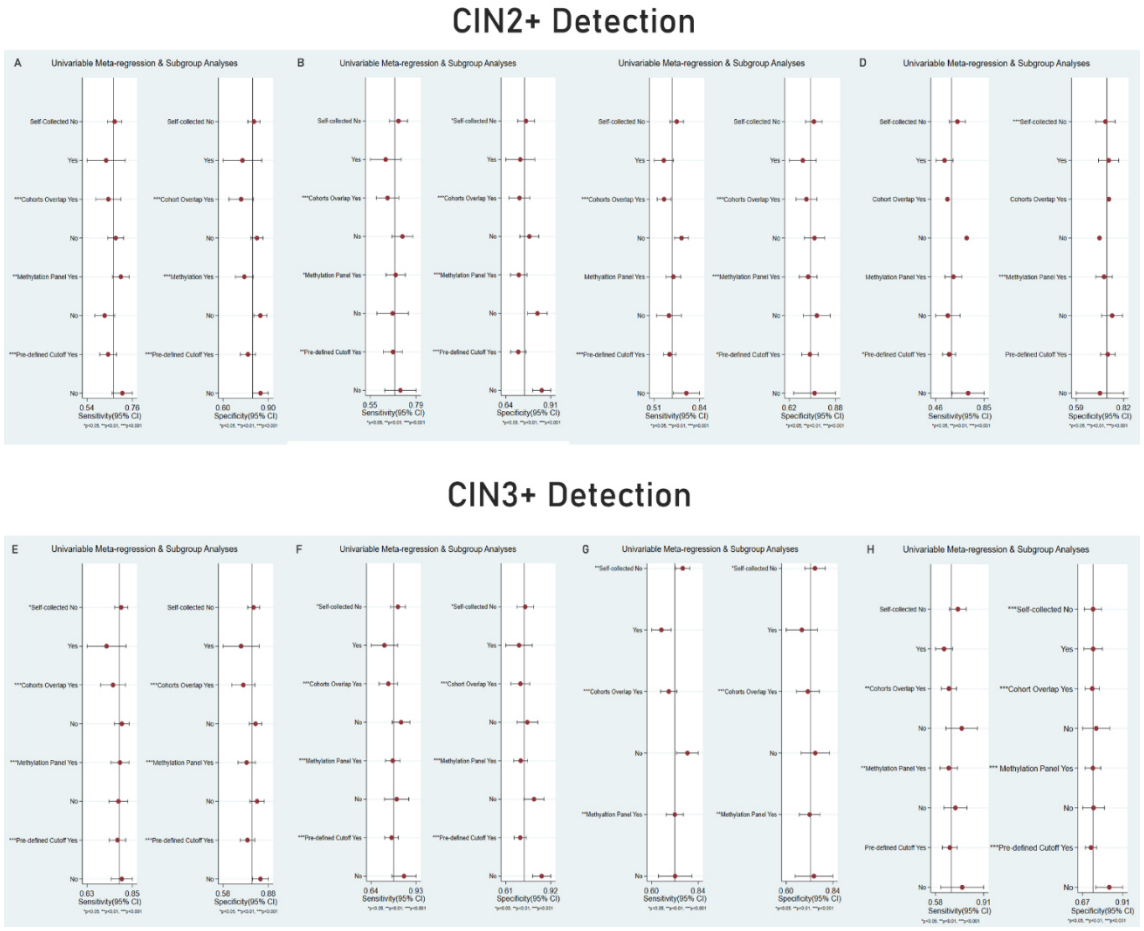
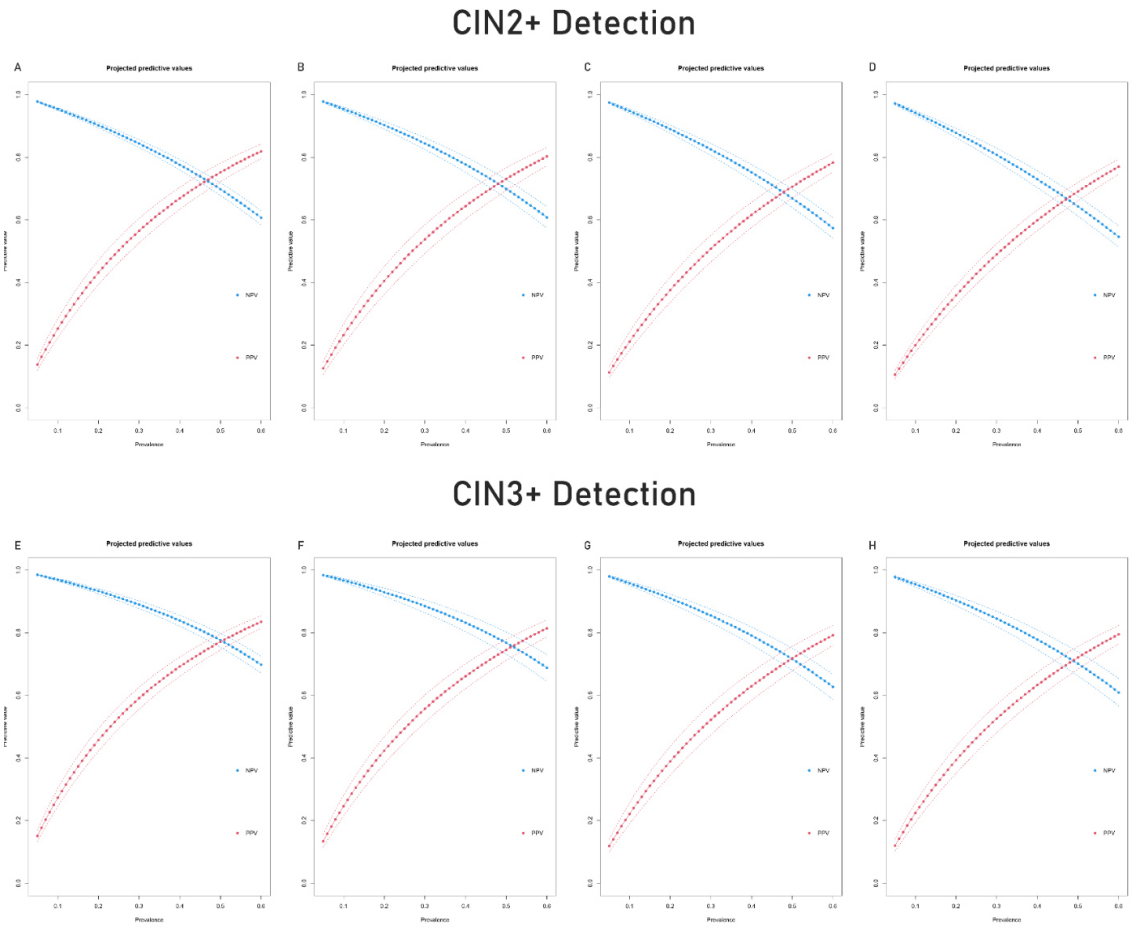

CIN2+ Detection
A Univariable Meta-regression & Subgroup Analyses
B Univariable Meta-regression & Subgroup Analyses
Univariable Meta-regression & Subgroup Analyses
D Univariable Meta-regression & Subgroup Analyses
CIN3+ Detection
E Univariable Meta-regression & Subgroup Analyses
F Univariable Meta-regression & Subgroup Analyses
G Univariable Meta-regression & Subgroup Analyses
H Univariable Meta-regression & Subgroup Analyses

Figure S4


CIN2+ Detection
A
Projected predictive values
B
Projected predictive values
C
Projected predictive values
D
Projected predictive values
NPV
PPV
Prevalence
CIN3+ Detection
E
Projected predictive values
F
Projected predictive values
G
Projected predictive values
H
Projected predictive values
NPV
PPV
Prevalence

Additional Methods

PubMed search strategy

For PubMed search was used the following strategy: ((("hpv positive" [title/abstract]) OR (“human papillomavirus” [title/abstract]) OR (carcinogenesis [MeSH Terms]) OR ("Cervical Intraepithelial Neoplasia"[Mesh]) OR ("Squamous Intraepithelial Lesions"[Mesh])) AND (("Cervix Uteri"[Mesh]) OR ("Cervix"[title/abstract])) AND ((nucleic acids [MeSH Terms]) OR (CpG islands [MeSH Terms]) OR ("DNA Methylation"[Mesh]) OR ("Epigenomics"[Mesh]) OR (methylation [MeSH Terms])) AND (("Triage"[Mesh]) OR ("Early Detection of Cancer" [Mesh]) OR (“Screening” [title/abstract]) OR ("referral" [title/abstract])))

Scopus search strategy

For Scopus search was used the following strategy: ((("hpv positive") OR (“human papillomavirus”) OR (carcinogenesis) OR ("Cervical Intraepithelial Neoplasia") OR ("Squamous Intraepithelial Lesions")) AND (("Cervix Uteri") OR ("Cervix")) AND ((nucleic acids) OR (CpG islands) OR ("DNA Methylation") OR ("Epigenomics") OR (methylation)) AND (("Triage") OR ("Early Detection of Cancer") OR (“Screening”) OR ("referral")))

Cochrane search strategy

For Cochrane search was used the following strategy: ((((“hpv positive") OR (“human papillomavirus”) OR (carcinogenesis) OR ("Cervical Intraepithelial Neoplasia") OR ("Squamous Intraepithelial Lesions of the cervix")) AND ((nucleic acids) OR (CpG islands) OR ("DNA Methylation") OR ("Epigeneomics") OR (methylation)) AND (("Triage") OR ("Early Detection of Cancer") OR (“Screening”) OR ("referral and consultation")))):ti,ab,kw

Standardisation of data collection

Data collected from included studies was performed according to a standardized extraction form. It included: Author, year of publication, country of study

population, type of study, number of samples analyzed, age range, cohort name, sample type, number of CIN0/controls, CIN1, CIN2, CIN3,and ICC, DNA methylation marker(s), method of methylation testing, type of cutoff, pre-defined cutoff, population overlap with other studies, number of true positives (TP), true negatives (TN), false positive (FP) and false negatives (FN) for methylation marker(s), cytology and HPV genotyping.

CHAPTER V –
Research Paper III

Research Paper III

Paper III – Performance of DNA methylation-based biomarkers in the cervical cancer screening program of northern Portugal: A feasibility study

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Sofia Salta, Leonardo Maia-Moço*, Helena Estevão-Pereira*, José Pedro Sequeira*, Renata Vieira, Carla Bartosch, Sara Petronilho, Paula Monteiro, Ana Sousa, Inês Baldaque, Jéssica Rodrigues, Hugo Sousa, Fernando Tavares, Rui Henrique#, Carmen Jerónimo#

Author's personal contribution:

Study design, sample preparation for molecular analyses, including DNA extraction from formalin-fixed paraffin embedding samples, bisulfite treatment and QMSP performance, data analysis and manuscript drafting.

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CANCER GENETICS AND EPIGENETICS

IJC International Journal of Cancer

Performance of DNA methylation-based biomarkers in the cervical cancer screening program of northern Portugal: A feasibility study

Sofia Salta[1,2] | Leonardo Maia-Moço[1] | Helena Estevão-Pereira[1] | José Pedro Sequeira[1,3] | Renata Vieira[1,4] | Carla Bartosch[1,4,5] | Sara Petronilho[1,4,5] | Paula Monteiro[4] | Ana Sousa[4,6] | Inês Baldaque[7] | Jéssica Rodrigues[8,9] | Hugo Sousa[6,7] | Fernando Tavares[10] | Rui Henrique[1,4,5] | Carmen Jerónimo[1,5]

[1]Cancer Biology & Epigenetics Group, Research Center of IPO Porto (CI-IPOP)/RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center (Porto.CCC), Porto, Portugal

[2]Doctoral Program in Molecular Pathology and Genetics, School of Medicine and Biomedical Sciences-University of Porto (ICBAS-UP), Porto, Portugal

[3]Master Program in Oncology, School of Medicine and Biomedical Sciences-University of Porto (ICBAS-UP), Porto, Portugal

[4]Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal

[5]Department of Pathology and Molecular Immunology, School of Medicine and Biomedical Sciences-University of Porto (ICBAS-UP), Porto, Portugal

[6]Molecular Oncology and Viral Pathology Group, Research Center of IPO Porto (CI-IPOP)/RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center (Porto.CCC), Porto, Portugal

[7]Virology Service, Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal

[8]Cancer Epidemiology Group, IPO Porto Research Center of IPO Porto (CI-IPOP)/RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center (Porto.CCC), Porto, Portugal

[9]Centre of Mathematics (CMAT), University of Minho, Campus de Gualtar, Braga, Portugal

[10]Administração Regional de Saúde do Norte, Porto, Portugal

Correspondence
Rui Henrique and Carmen Jerónimo, Portuguese Oncology Institute of Porto (IPO Porto), Rua Dr. António Bernardino de Almeida; 4200-072 Porto, Portugal.
Email: henrique@ipoporto.min-saude.pt (R. H.) and carmenjeronimo@ipoporto.min-saude.pt (C. J.)

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Abstract

Cervical cancer remains a health concern. Effective screening programs are critical to reduce the incidence and mortality. High-risk HPV (hr-HPV) testing as primary screening tool discloses high sensitivity but suboptimal specificity. Adequate triage tests to reduce unnecessary colposcopy referrals and overdiagnosis/overtreatment are crucial. Hence, we aimed to validate a panel of DNA methylation-based markers as triage test for women hr-HPV+ in the population-based Regional Cervical Cancer Screening Program of Northern Portugal. Firstly, *CADM1*, *MAL*, *FAM19A4* and *hsa-miR124-2* promoter methylation levels were assessed by multiplex QMSP in a testing set of 402 FFPE tissue samples (159 normal samples and 243 cervical lesions, including 39 low-grade intraepithelial squamous lesions [LSIL], 59 high-grade intraepithelial

Abbreviations: ADC, adenocarcinoma; AIS, adenocarcinoma in situ; ASC-US, atypical squamous cells of undetermined significance; AUC, area under the curve; CI, confidence interval; CIN, cervical intraepithelial neoplasia; FFPE, formalin-fixed paraffin-embedded; Hr-HPV, high risk human papillomavirus; HSIL, high-grade squamous intraepithelial lesions; HSIL+, HSIL or worse lesions; LSIL, low-grade squamous intraepithelial lesions; mQMSP, multiplex quantitative methylation-specific PCR; NILM, negative for intraepithelial lesion or malignancy; NPV, negative predictive value; PCA, principal component analysis; PPV, positive predictive value; ROC, receiver operator characteristic; SCC, squamous cell carcinoma; SNS, Portuguese National Health Service.

Leonardo Maia-Moço, Helena Estevão-Pereira and José Pedro Sequeira contributed equally to our study.

squamous lesions [HSIL] and 145 cancerous lesions). Then, preliminary validation was performed in 125 hr-HPV+ cervical scrapes (including 59 normal samples, 30 LSIL, 34 HSIL and 2 cancerous lesions). Higher *MAL*^{me}, *FAM19A4*^{me} and *hsa-miR124-2*^{me} methylation levels were disclosed in histological HSIL or worse (HSIL+) in testing set. Individually, markers depicted over 86% specificity for HSIL+ detection. In validation set, all these genes significantly differed between histological HSIL+ and low-grade squamous intraepithelial lesions or less. In combination, these markers reached 74% specificity and 61% sensitivity for identification of histological HSIL+. We concluded that host gene methylation might constitute a useful referral triage tool of hr-HPV+ women enrolled in the Cervical Cancer Screening Program of Northern Portugal.

KEYWORDS

biomarkers, cervical cancer screening, colposcopy referral, DNA methylation, triage

What's new?

For cervical cancer screening, high-risk HPV (hr-HPV) testing is highly sensitive, but for those who test positive, a more specific test is needed to reduce unnecessary colposcopy. Here, the authors validated a panel of DNA methylation-based markers to screen hr-HPV+ women. They measured promoter methylation of four genes, CADM1, MAL, FAM19A4, and hsa-miR124-2, in cervical samples including normal tissue, low- and high-grade squamous intraepithelial lesions (LSIL and HSIL) and cancerous lesions. The markers showed 74% specificity for HSIL or cancer, indicating that DNA methylation could be a useful triage tool for hr-HPV+ women.

1 | BACKGROUND

Cervical cancer is the fourth most incident and most deadly cancer among women worldwide.[1] In Portugal, it stands as the 9th and 12th most incident neoplasm among women, nationwide or in the Northern Region, respectively, in 2018.[2] Persistent high-risk human Papillomavirus (hr-HPV) infection is the main cause of cervical cancer, accounting for vast majority of cases.[3] The implementation of cervical cancer screening programs led to a decrease in the incidence and mortality across many countries.[4-6] The Regional Cervical Cancer Screening Program of Northern Portugal is an organized, population-based, fully supported by the Portuguese National Health Service (SNS), which started in 2009 and progressively extended to the whole region.[7] The distribution of hr-HPV genotypes in this screened population was previously published by us.[8]

European guidelines for cervical cancer screening recommend hr-HPV-based screening owing to its high sensitivity and negative predictive value (NPV).[9-12] However, due to the high incidence of transient hr-HPV infections, specificity of hr-HPV testing is limited, especially among young women, in which hr-HPV infection is very frequent but seldom persists.[13-15] Thus, several triage methods have been proposed to reduce unnecessary colposcopy referrals, including cytological examination,[15] eventually complemented with dual immunohistochemistry for p16 and Ki67 (eg, CINtec PLUS),[16] and DNA methylation-based markers.[17] The latter constitute a particularly promising triage strategy in this setting due to their good performance, enabling fully automated molecular testing for cervical cancer screening, and the possibility of promoting self-collection strategies which might increase screening compliance.[18-20]

Several DNA methylation-based markers have been proposed over the years, basically comprising DNA methylation of host and/or HPV genes.[18,19,21] Among these, *CADM1*, *MAL*, *FAM19A4* and *hsa-miR124-2* are the most extensively studied,[17,22-32] including in a population-based screening scenario.[17,19,27-31,33] These genes combined in a panel may reach 70% sensitivity for detection of high-grade lesions, with high specificity.[17,23,25-33] Nonetheless, most studies were reported in the Dutch population,[17,22-32,34] and validation in other populations is rather limited, especially regarding *CADM1* and *MAL*.[18,19,35]

As part of an ongoing effort to streamline referral to colposcopy and implement full testing automation, we aimed to validate the potential of *CADM1*, *MAL*, *FAM19A4* and *hsa-miR124-2* promoter methylation in the Northern Portugal population-based cervical cancer screening program. For this purpose, two sets of samples were selected: one composed of formalin-fixed paraffin-embedded (FFPE) samples to assess the methylation status of the target genes in cervical lesions from the Portuguese population, allowing for selection of those displaying the best biomarker performance; the second sample set comprised cervical scrapes from women testing positive for hr-HPV in the screening procedures, to assess the potential usefulness of those biomarkers as triage test.

2 | MATERIAL AND METHODS

2.1 | Patients and samples selection

A total of 243 FFPE tissue samples (Cohort #1) of cervical lesions from patients aged 21-88 years old, diagnosed at the Portuguese Oncology Institute of Porto (IPO Porto) between 2008 and 2020 were selected retrospectively from the archives of the Department of Pathology, comprising 39 low-grade squamous intraepithelial lesions (LSIL), 59 high-grade squamous intraepithelial lesions (HSIL), 70 squamous cell carcinomas (SCC), 21 adenocarcinomas in situ (AIS) and 54 adenocarcinomas (ADC). All the lesions were classified according WHO Classification of Tumors of Female Reproductive Organs (4th edition).[36] For squamous precursor lesions "The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions" was used.[37] All cases corresponded to surgical specimens as patients with only biopsy available were excluded. Additionally, 159 FFPE tissue samples (Cohort #2) of normal cervix were selected from women aged 38-83 years old, without cervical cancer nor any precursor lesion, which were submitted to gynecological surgery (hysterectomy) at IPO Porto. All diagnoses were reviewed by a dedicated gynecopathologist. Selected FFPE blocks were cut for DNA extraction and were stained with H&E to identify and mark the area of interest, ensuring its presence across the sections used for methylation analysis.

A total of 125 cervical scrapes (Cohort #3) collected in methanol-based, buffered preservative solution (ThinPrep PreservCyt) from subjects aged 24-61 years old, enrolled in the Regional Cervical Cancer Screening Program of Northern Portugal, between March and May 2019 were used for validation (Cohort #3). This is an organized, population-based, screening program offered to women registered in primary care. Women aged between 25 and 60 years are invited to attend a consultation with their general practitioner, in which a cervical scrape is collected in ThinPrep PreservCyt. Women with previous history of cervical cancer, hysterectomy or those who did not yet started sexual activity are excluded. All samples are sent to the reference laboratory (at IPO Porto) and tested for hr-HPV using the Anyplex II HPV HR Detection kit (Seegene, Seoul, Korea).[8] This method, already validated for use in cervical cancer screening,[38] allows for simultaneous detection and genotyping of 14 hr-HPV genotypes (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66 and HPV-68). For all samples, DNA extracted for hr-HPV testing is stored. Cytopathological examination of all cases, performed by dedicated cytopathologists, is the currently used triage method in this screening program. According to the screening algorithm, cases with a result worse than negative for intraepithelial lesion or malignancy (NILM) and positive for hr-non-HPV16/18, are referred for colposcopy; hr-HPV16/18 positive cases are directly referred to colposcopy, irrespective of the result of cytological examination. Cases with hr-HPV positivity (non-HPV16 nor HPV18) and NILM repeat hr-HPV testing after a year. The hr-HPV negative cases are invited to repeat cervical scrape after 5 years. At present, the coverage of the program is about 40% of target population and it is free of charge, as well as the follow-up and eventual treatment, since it is funded by the SNS. According to our data, the rate of hr HPV tests considered insufficient is 0.44% and inconclusive tests account for only 0.04%.[8]

Cohort #3 patients were selected, retrospectively, if tested positive for hr-HPV and were referenced for colposcopy. According to the results of the colposcopy and/or biopsy, these cases were classified as: normal (negative biopsy result or no lesion observed in the colposcopy [n = 59]), LSIL (n = 30), HSIL (n = 34), AIS (n = 1) and SCC (n = 1). Patients that were not referred for colposcopy were excluded from analysis. Methylation testing was performed using the leftover samples primarily used for diagnosis. Relevant clinical data is depicted in Table S1.

2.2 | Methylation analyses

From all FFPE samples of Cohorts #1 and #2, a 4-μm section was cut before and after the 8-μm sections used for DNA extraction, and stained with routine hematoxylin-eosin staining, to ensure the target lesions were present across all sections. Lesional areas were delimited and macrodissected from 8 to 10 consecutive 8-μm sections. Nuclei acid extraction was performed using FFPE RNA/DNA Purification Plus Kit FFPE purification (Norgen Biotek, Thorold, Canada), according to manufacturer's instructions. DNA extracted from FFPE was quantified using NanoDrop Lite spectrophotometer (NanoDrop Technologies, Wilmington, Delaware) and stored at −20°C until further use.

From cervical scrapes (Cohort #3) we used the leftover of the DNA primarily used for diagnosis, which was extracted in an automated system, as previously described.[8] All the samples were stored at −80°C until methylation assays have been performed.

Bisulfite treatment was performed for all samples using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, California), according to manufacturer's instructions. For control purposes, 1 μg of Human HCT116 DKO Methylated DNA (positive control [Zymo Research, Orange, California]) and 1 μg Human HCT116 DKO Non-Methylated DNA (negative control [Zymo Research, Orange, California]) were bisulfite-treated and eluted in 20 μL of sterile distilled water. For FFPE samples, when available, 1 μg of DNA was treated and eluted in 60 μL of sterile distilled water. For the smaller DNA amounts, elution volumes were adjusted. For cervical scrapes, 20 μL of DNA was bisulfite-treated and eluted in 15 μL of sterile distilled water. Additionally, 8 μL treated cervical scrape DNA and both controls were preamplified using SsoAdvanced PreAmp Supermix (Bio-Rad, Hercules, California) according to manufacturer's recommendations. The final reaction was diluted 1:5 in sterile distilled water. Methylation analysis was performed using 1 μL of DNA as template in Multiplex Quantitative Methylation-specific PCR (mQMSP). Primers and probes targeting the methylated genome sequence are listed in Table S2. *β-Actin* was used as reference gene. Reactions were carried out in 384-well plates using QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Perkin Elmer, California). mQMSP reactions used Xpert Fast Probe (GRISP, Porto, Portugal; Table S3). All samples were run in triplicate

and a negative control and standard curve (generated from the positive control) were included for quantification and to ascertain PCR efficiency for each plate. The samples were considered validated for β-actin analysis when Ct ≤ 30, for FFPE samples, or Ct ≤ 25, for cervical scrapes samples. For FFPE samples, target methylation was considered for analysis if Ct ≤ 40. For cervical scrapes, only target genes' methylation with Ct ≤ 30 was considered for analysis. Target methylation with higher Ct was considered negative for methylation (quantity mean = 0). Relative methylation levels were determined as the ratio of the mean quantity for each gene and the mean quantity of the reference gene, multiplied by 1000 for easier tabulation.

2.3 | Statistical analysis

Principal component analysis (PCA) was performed for methylation variables according to the diagnosis using R software v.4.0.1. Comparisons between groups were performed using Mann-Whitney tests for continuous variables. To assess biomarker performance, receiver operator characteristic (ROC) curves were constructed for each gene and the area under the curve (AUC) was calculated for detection of HSIL+ (HSIL or worse according to the histological result). According to ROC curve analysis, the cut-off to categorize samples as methylated or unmethylated was established, maximizing Youden's Index (sensitivity + specificity − 1).[39,40] Furthermore, specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), accuracy and the corresponding 95% confidence intervals (CI) were calculated for detection of HSIL+ lesions based on the histological diagnosis (histological HSIL+). For the combination of biomarkers, samples were considered positive if at least one of the individual biomarkers was positive. For cervical scrape, additional Multiple ROC Curve analysis was performed as previously described.[41] Two-tailed P-values were derived from statistical tests, using a computer-assisted program (SPSS Version 26.0, Chicago, Illinois), and results were considered statistically significant at $P < .05$. Graphs were assembled using GraphPad 8 Prism (GraphPad Software, The United States).

3 | RESULTS

3.1 | Distribution of methylated gene promoters among cervical lesions in tissue samples

PCA was performed to better understand how samples' variance might be explained by *CADM1*^{me}, *MAL*^{me}, *FAM19A4*^{me} and *hsa-miR124-2*^{me} promoter methylation levels. PC1 was composed by *MAL*^{me}, *FAM19A4*^{me} and *hsa-miR124-2*^{me} promoter methylation and explained 63.51% of data variance, whereas PC2, composed by *CADM1*^{me} promoter methylation, explained 23.36% of data variance. Although there was not a clear separation of all populations, invasive lesions (SCC and ADC) clearly separated from Normal tissue and LSIL, then followed by HSIL and AIS (Figure S1). Additionally, HSIL+ lesions

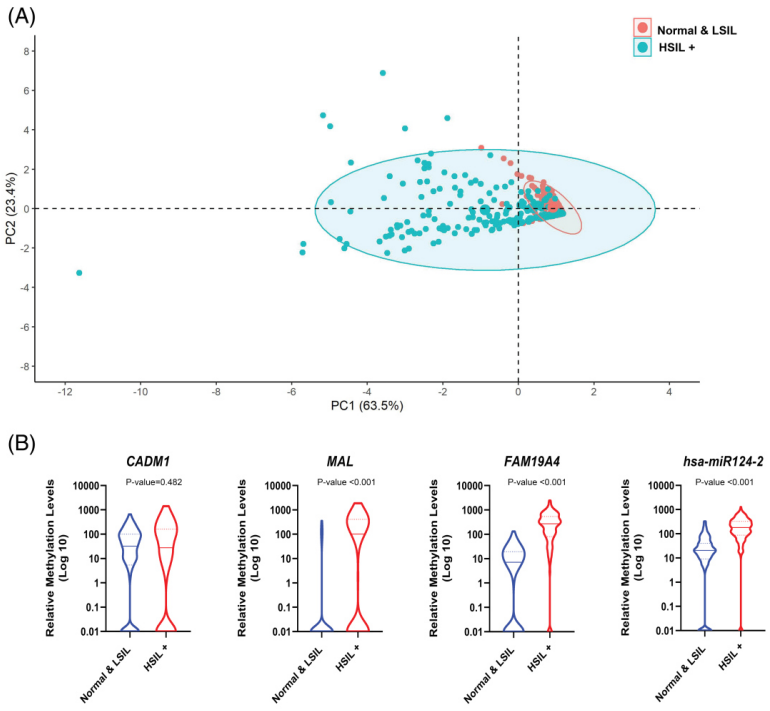

FIGURE 1 Methylation distribution in cervical FFPE samples. (A) Principal component analysis of cervical samples included in our study according to histological diagnosis. Scatterplot of samples according to methylation levels. (B) Violin plots for *CADM1*, *MAL*, *FAM19A4* and *hsa-miR124-2* methylation levels. Dashed lines indicate the interquartile range and horizontal line the median of methylation levels distribution [Color figure can be viewed at wileyonlinelibrary.com]

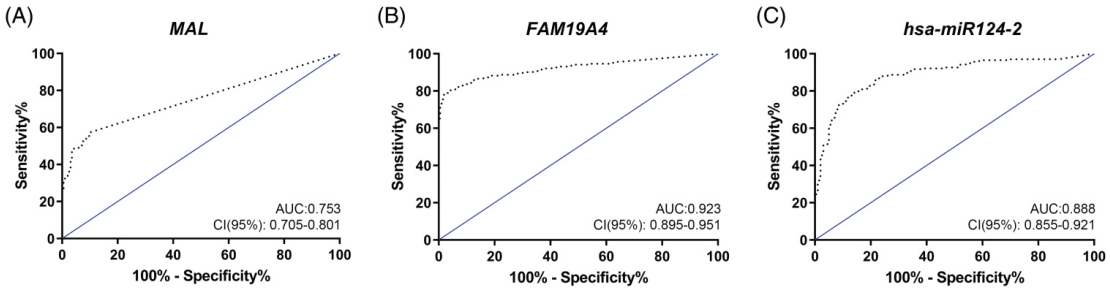

FIGURE 2 Receiver operating characteristic (ROC) curves depicting biomarker performance for detection of HSIL or worse lesions of the *MAL* (A), *FAM19A4* (B) and *hsa-miR124-2* (C) methylation levels in cervical FFPE tissues [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Performance of promoter gene methylation as biomarkers in FFPE samples for detection of histological diagnosis of high-grade intraepithelial lesions or worse

Gene	Normal and LSIL, n = 198, n (%)[a]	HSIL +, n = 204, n (%)[a]	Specificity % (CI 95%)	Sensitivity % (CI 95%)	PPV % (CI 95%)	NPV % (CI 95%)	Accuracy (CI 95%)
MAL^{me}	20 (10.10)	117 (57.35)	89.90 (85.70-94.10)	57.37 (50.57-64.14)	85.40 (79.49-91.31)	67.17 (61.52-72.82)	73.38 (69.06-77.70)
FAM19A4^{me}	7 (3.535)	164 (80.39)	96.46 (93.89-99.04)	80.39 (74.94-85.84)	95.91 (92.94-98.88)	82.68 (77.80-87.56)	88.31 (85.17-91.45)
hsa-miR124-2^{me}	26 (13.13)	160 (78.43)	86.87 (82.16-91.57)	78.43 (72.79-84.04)	86.02 (81.04-91.00)	79.63 (74.26-85.00)	82.59 (78.88-86.25)

[a]Number of positives cases and percentage of the total.
Abbreviations: CI, confidence interval; HSIL+, high-grade squamous intraepithelial lesions or worse; LSIL, low-grade squamous intraepithelial lesions; NPV, negative predictive value; PPV, positive predictive value.

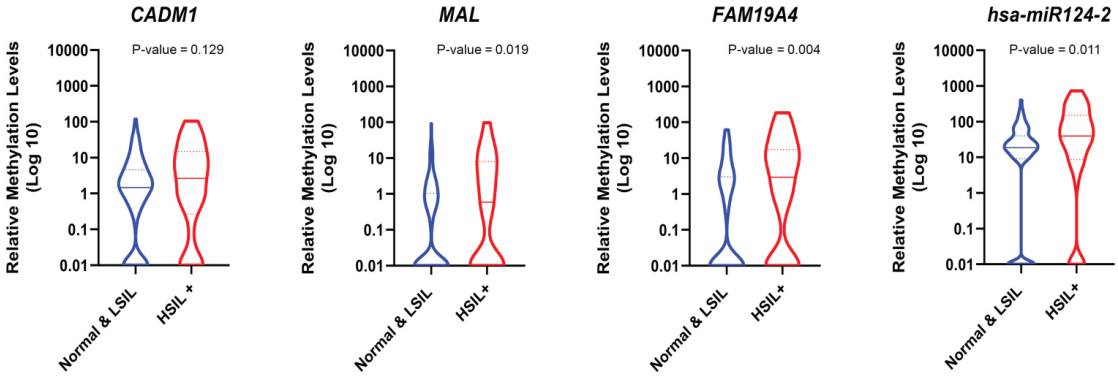

FIGURE 3 Violin plots for *CADM1*, *MAL*, *FAM19A4* and *hsa-miR124-2* promoter's methylation in cervical scrapes. Dashed lines indicate the interquartile range and horizontal line the median of methylation levels distribution [Color figure can be viewed at wileyonlinelibrary.com]

disclosed higher dispersion across PC1 compared to Normal and LSIL (Figure 1A). However, dispersion across PC2 was similar between groups.

When analyzing the methylation levels of each gene individually, except for *CADM1*^{me} ($P = .428$), methylation levels in HSIL+ were significantly higher than in the Normal and LSIL group ($P < .001$; Figure 1B).

3.2 | Biomarkers' performance in FFPE samples

ROC curve analysis assessed the performance of methylation markers in identifying histological HSIL+ (Figure 2). The best cut-off for each gene was defined (0.3137 for *MAL*^{me}, 54.494 for *FAM19A4*^{me} and 77.638 for *hsa-miR124-2*^{me}), resulting in an AUC over 0.75. Moreover, all genes depicted a specificity above 85% (Table 1), with the best

TABLE 2 Performance of promoter gene methylation as biomarkers in cervical scrapes for detection of histological diagnosis of high-grade intraepithelial lesions or worse

Gene	Normal and LSIL, n = 89, n (%)[a]	HSIL +, n = 36, n (%)[a]	Specificity % (CI 95%)	Sensitivity % (CI 95%)	PPV % (CI 95%)	NPV % (CI 95%)	Accuracy (CI 95%)
MAL^{me}	9 (10.11)	14 (38.89)	89.89 (83.62-96.15)	38.89 (22.96-54.81)	60.87 (40.92-80.82)	78.43 (70.45-86.41)	75.20 (67.63-82.77)
FAM19A4^{me}	16 (17.98)	18 (50.00)	82.02 (74.04-90.00)	50.00 (33.67-66.33)	52.94 (36.16-69.72)	80.22 (72.04-88.40)	72.80 (65.00-80.60)
hsa-miR124-2^{me}	11 (12.36)	15 (41.67)	87.64 (80.80-94.48)	41.67 (25.56-57.77)	57.69 (38.70-76.68)	78.79 (70.73-86.84)	74.40 (66.75-82.05)
FAM19A4^{me}/MAL^{me}	19 (21.35)	20 (55.56)	78.65 (70.14-87.16)	55.56 (25.56-71.79)	51.28 (35.59-66.97)	81.40 (73.17-89.62)	72.00 (64.13-79.87)
hsa-miR124-2^{me} /MAL^{me}	18 (20.22)	20 (55.56)	79.78 (71.43-88.12)	55.56 (25.56-71.79)	52.68 (36.76-68.51)	81.61 (73.47-89.75)	72.80 (65.00-80.60)
FAM194^{me}/hsa-miR124-2^{me}	21 (23.60)	20 (55.56)	76.40 (67.58-85.23)	55.56 (25.56-71.79)	48.78 (33.48-64.08)	80.95 (72.55-89.35)	70.40 (62.40-78.40)
All 3-genes	22 (24.72)	22 (61.11)	74.16 (65.06-83.25)	61.11 (45.19-77.04)	48.89 (34.28-63.49)	82.50 (74.17-90.83)	70.40 (62.40-78.40)

[a]Number of positives cases and percentage of the total.
Abbreviations: CI, confidence interval; HSIL+, high-grade squamous intraepithelial lesions or worse; LSIL, low-grade squamous intraepithelial lesions; NPV, negative predictive value; PPV, positive predictive value.

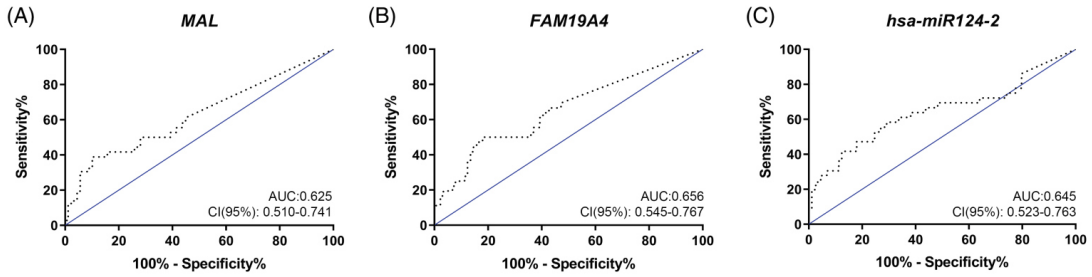

FIGURE 4 ROC curves depicting biomarker performance for detection of HSIL or worse lesions of *MAL* (A), *FAM19A4* (B) and *hsa-miR124-2* (C) methylation levels in cervical scrapes [Color figure can be viewed at wileyonlinelibrary.com]

accuracy, specificity and sensitivity (88.31%, 96.46% and 80.39%, respectively) achieved by *FAM19A4^{me}*.

3.3 | Biomarker performance in cervical scrapes

Methylation levels were assessed in cervical scrapes from hr-HPV positive women enrolled in the Northern Portugal Cervical Cancer Screening Program. Among the 125 cases tested, all were considered valid for analysis as β-actin disclosed Ct ≤ 25. For target genes, no methylation (quantity mean = 0) was disclosed in 29 cases for *CADM1^{me}*, 63 cases for *MAL^{me}*, 58 for *FAM19A4^{me}* and 23 for *hsa-miR124-2^{me}*. Only 7 of these cases had no methylation for all 4 genes simultaneously.

FAM19A, *MAL* and *hsa-miR124-2* promoter methylation levels significantly differed between the histological HSIL+ group and the Normal and LSIL group, in line with the results observed in FFPE tissues (Figure 3).

Studied genes disclosed high specificity (range: 82.02%-89.89%; Table 2), with an AUC above 60% (Figure 4), nevertheless sensitivity was low, ranging between 38.89% and 50.00%. When markers were combined in two-gene panels, sensitivity reached 55.56% with specificity ranging from 76.40% to 78.65%. The 3-genes combination achieved 61.11% sensitivity and 74.16% specificity (Table 2). To increase the statistical power, multiple ROC curve analysis was performed for different gene panels with 1000 repetitions. Nonetheless, a slight decrease in biomarkers performance was observed (Table S4).

4 | DISCUSSION

In our study we aimed to validate the promoter methylation of four genes (*CADM1^{me}*, *MAL^{me}*, *FAM19A4^{me} and hsa-miR124-2^{me}*) as candidate triage tests for hr-HPV positive women, in the screening program of Northern Portugal. Interestingly, *MAL^{me}*, *FAM19A4^{me}* and *hsa-miR124-2^{me}* promoters were found differentially methylated between

the HSIL+ group and the Normal and LSIL group, allowing for highly specific detection of more advanced lesions in tissue samples. Although a reduction in sensitivity was apparent in cervical scrapes, specificity was higher than 70%, for all combinations. Globally, our results are comparable to those previously reported,[19,42] confirming the performance of these biomarkers in our screening population.

The major limitation of our study is the restricted number of samples in Cohort #3, impairing a more robust assessment of biomarker performance in cervical scrapes. To overcome this limitation, multiple ROC Curve analysis was performed. Nonetheless, it should be taken in consideration that we carried out the analysis in the same samples which were used for HPV testing. This allied to the storage-time of the low concentrated DNA samples may eventually limit biomarker performance, negatively influencing our results.

Notwithstanding, the results of our study confirm the potential of host-genes promoter methylation for triage of hr-HPV+ cases in cervical cancer screening programs. We developed a "homemade" approach for assessing methylation in cervical scrapes which has, nonetheless, some limitations. The major technical limitation is the need for a preamplification step previously to mQMSP analysis.

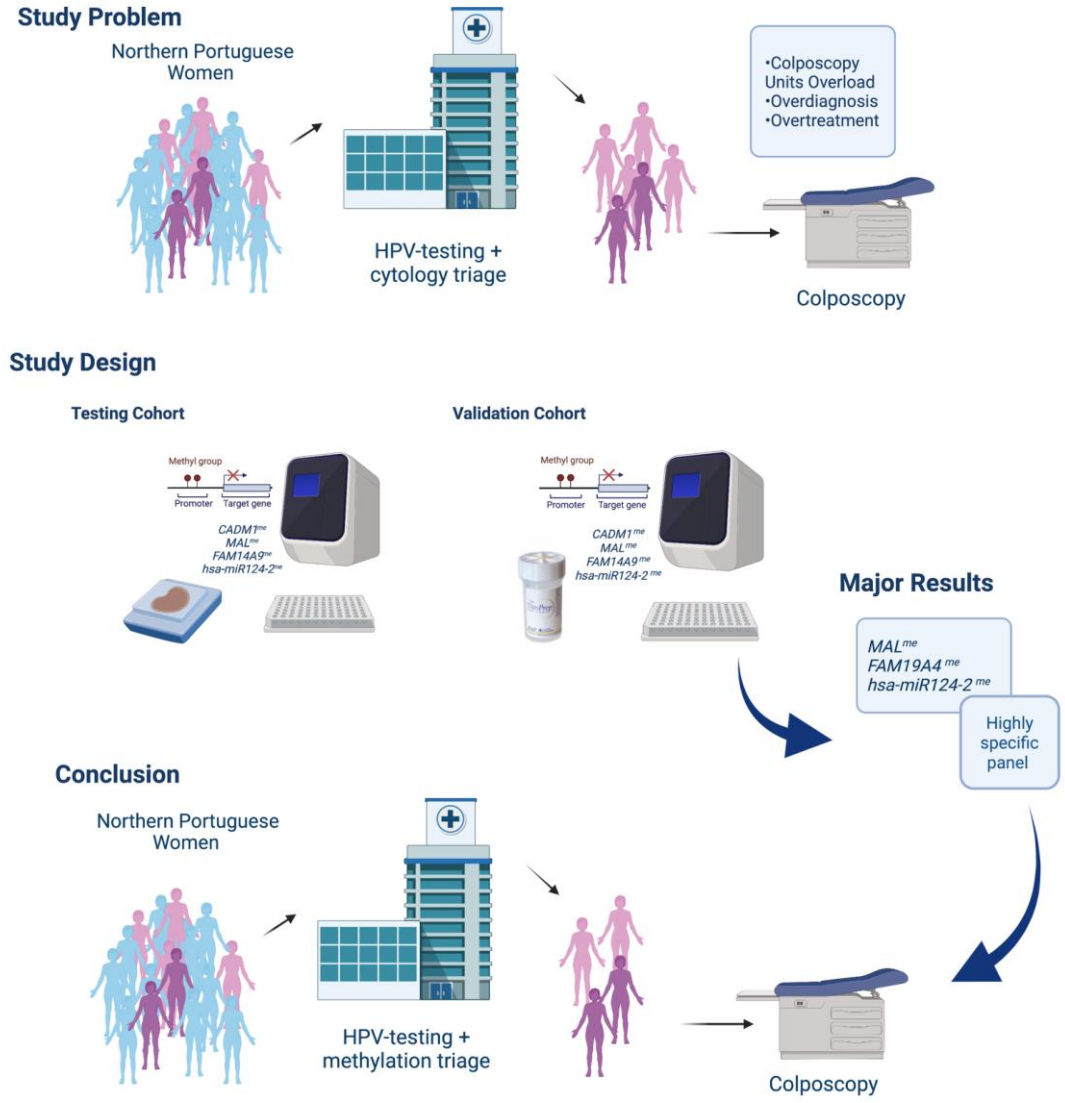

FIGURE 5 Flowchart of study design and major findings. Created with BioRender.com [Color figure can be viewed at wileyonlinelibrary.com]

DNA preamplification might also explain the decrease in biomarkers performance in cervical scrapes compared to FFPE tissue samples, since this methodology is more prone to increase bias in the results.[43] The multiplex reaction of preamplification is more likely to generate false positive results, eventually influencing cut-off determination and, consequently, biomarker performance. New methodologies, such as digital PCR, are likely to increase sensitivity of the candidate biomarkers without affecting specificity and circumventing the need for DNA preamplification.[43] Nevertheless, our strategy has also some advantages, including the use of a faster bisulfite treatment (EZ DNA Methylation-Gold Kit [~3-4 hr] instead of EZ DNA Methylation Kit [~16 hr]), enabling a more cost-effective test for cervical cancer screening programs.

The results observed for *CADM1*^{me} were disappointing as methylation levels did not significantly differ between controls and lesions, indicating that this is not a good candidate for triage in the screening context. Remarkably, Yin et al reported differential *CADM1*^{me} between cervical intraepithelial neoplasia (CIN) 3+ lesions and CIN1, but not between CIN1− and CIN2.[44] Moreover, a similar methylation distribution was reported by Van Zummeren et al, with CIN2 lesions disclosing methylation levels similar to CIN1 lesions.[45]

The variation of biomarker performance observed in our study, when compared to previously reported studies, might be partially explained by differences in target populations. However, it should be recalled that these are not the only cause for test performance variability. Indeed, a multicenter study that analyzed the commercially available kit QIAsure Methylation Test (Qiagen), which includes *FAM19A4*^{me} and *hsa-miR124-2*^{me} promoter methylation, reported a significant variability in sensitivity and specificity across different centers in HPV-based screening programs. This may be partially explained by the different sample collection media, as well as different DNA extraction methods,[35] which does not occur in the setting of our screening program. In our study, all the samples were valid for methylation assessment (Ct ≤ 25 for β-actin). This was likely due to the use of the same DNA sample extracted for hr HPV genotyping, which very rarely is deemed insufficient (0.44% in our experience[8]). We believe that this strategy reduces the rate of invalid tests since DNA extraction is used for both procedures, contrarily to QIAsure Methylation Test which requires a second extraction procedure.[35] This probably accounts for the 10% rate of invalid tests disclosed in that multicenter study, although with high variability among participating centers (3%-24%).[35]

Furthermore, Bonde et al reported a reduction in biomarker performance with QIAsure Methylation Test in samples from routine screening programs (Slovenian cohort). Remarkably, the range of sensitivity and specificity achieved for detecting CIN2+ in cervical smears was similar to ours (HSIL+ lesions).[35] Likewise, Dippman et al reported 63.3% sensitivity and 67.4% specificity for CIN2+ detection using the QIAsure Methylation Test.[42]

Thus, the inclusion of *MAL*^{me} in the gene panel (as we did) results in better specificity, although at the cost of lower sensitivity. Nonetheless, considering the context of a screening program, with surveillance and regular testing of previously negative cases every 5 years, as well as the need to reduce unnecessary referral to colposcopy, the option to increase specificity seems advantageous and favors the use of the 3-gene panel. In the case of Northern Portugal, colposcopy is presently the bottleneck of the screening program. Furthermore, considering that screening starts at age 25, with hr-HPV testing, overdiagnosis and overtreatment are also important concerns. Furthermore, the specificity of cytology as triage test is inferior since the observer is aware of hr-HPV positivity and is biased towards identification of lesion. Thus, in the context of our screening program, methylation would provide a more specific and more unbiased test, decreasing unnecessary referral to colposcopy, as well as overdiagnosis and overtreatment (Figure 5). Notwithstanding, hr-HPV positive women with a methylation negative result might be offered closer surveillance with repetition of HPV and/or methylation test within 1-2 years. Although this algorithm might be less cost-effective in a short-term, a fast time-response for high-grade lesions and the reduction of unnecessary medical procedures will increase cost-effectiveness of the screening program. Moreover, methylation as triage test identified risk for CIN3+ or cervical cancer similar to cytology and HPV genotyping in longitudinal studies, with the major advantage of allowing for assessment in self-collected samples.[33,46,47] Nonetheless, it should be acknowledged that different strategies might be adopted in other regions/countries, aiming at increased colposcopy referral and cervical cancer diagnosis. In these situations, methylation-based triage might be less cost-effective than extended genotyping and cytology.

In conclusion, our study confirms gene promoter methylation of selected genes as a promising triage tool for assessment of hr-HPV+ cases, specifically in the context of the population-based program of Northern Portugal. The use of a 3-gene panel might aid in decreasing referral for colposcopy. Indeed, combined with existing screening tools, this methylation panel might reduce overtreatment and overdiagnosis, improving cost-effectiveness of our regional cervical cancer screening program.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Our study was approved by the institutional review board of IPO Porto (Comissão de Ética para a Saúde - CES-371/2017). All samples

derived from routinely archived material (FFPE tissues from surgical specimens or DNA from the cervical scrapes), which were used after anonymization. Thus, according to Portuguese law, consent to use the samples was waived.

ORCID

Carmen Jerónimo https://orcid.org/0000-0003-4186-5345

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Supporting Information

Available at

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Appendix S1

Supplementary Material

Title: Performance of DNA methylation-based biomarkers in the Cervical Cancer Screening Program of Northern Portugal: a feasibility study

Sofia Salta, Leonardo Maia-Moço[*], Helena Estevão-Pereira[*], José Pedro Sequeira[*], Renata Vieira, Carla Bartosch, Sara Petronilho, Paula Monteiro, Ana Sousa, Inês Baldaque, Jéssica Rodrigues, Hugo Sousa, Fernando Tavares, Rui Henrique[#], Carmen Jerónimo[#]

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Supplementary Table 1 – Clinicopathological data of cervical smears patients' cohort

Clinicopathological Features	Cervical Lesions	Normal Samples
N	66	59
Age median (range)	40 (24-61)	39 (24-60)
HPV[1] Genotype (n)		
HPV-16	11	7
HPV-18	3	---
Other hr-HPV[2]	25	28
Multiple Infection including HPV-16	11	7
Multiple Infection including HPV-18	2	4
Multiple Infection including other hr-HPV	14	14
Cytological Diagnosis (n)		
NILM[3]	---	6
ASC-US[4]	20	39
ASC-H[5]	15	---
LSIL[6]	11	11
HSIL[7]	20	---
AGC[8]	---	1
Unsatisfactory	---	2
Histological Diagnosis (n)		
Normal	---	19
Cervicitis	---	6
LSIL[6]	30	---
HSIL[7]	13	---
HSIL[7] (CIN[9] 2)	9	---
HSIL[7] (CIN[9] 3)	12	---
SCC[10]	1	---
AIS[11]	1	---
N.A.[12#]	---	34

[1] HPV – Human Papilloma Virus; [2] hr-HPV – high-risk HPV; [3] NILM – Negative for Intraepithelial Lesion or Malignancy; [4] ASC-US – Atypical Squamous Cell of Undetermined Significance; [5] ASC-H – Atypical Squamous Cells, Cannot Rule Out High Grade Squamous Intraepithelial Lesion; [6] LSIL – Low grade Squamous Intraepithelial Lesion; [7] HSIL – High grade Squamous Intraepithelial Lesion; [8] AGC – Atypical Glandular Cells; [9] CIN – Cervical Intraepithelial Neoplasia; [10] SCC – Squamous Cell Carcinoma; [11] AIS – Adenocarcinoma *in situ*; [12] N.A. – Not available; [#] – Women who have undergone colposcopy examination, but no lesion was found and, therefore, biopsy was not performed.

Supplementary Table 2 – Sequence of primers and probes used in this study.

Gene	Primer Forward Sequence	Primer Reverse Sequence	Probe Sequence
FAM19A4	AGTCGGGCGGTTCGGTTA	CCAAAACGACGCGCAACTA	FAM – CGAACCCGCCGCGAAACG–BHQ1
hsa-miR-124-2	GGTAATTAATTTGGATTTACGTCGTTAT	GTAAAAATATAAACGATACGTATACCTACG	HEX–TCTCCATACCATTTACAACACACGCCTAAA– BHQ1
CADM1 – M18	ATTTTATTAGTTGTTCGTTCGGGT	CTCGACAACACTACACTCGCC	FAM – ACCTCAAACTAACGACGTTAACTACCTCCG – BHQ1
MAL – M1	GCGTAGTATTAAGTAGAGAGGTTCG	AATAAAAAATAAAACCGACCGC	HEX – ACTAAACCGACGCTAATTCGACGCT – BHQ1
β-ACT	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	Cy5 - ACCACCACCCAACACACAATAACAAACACA – BHQ2

Supplementary Table 3 – Primers and Probes concentrations in PCR reactions used in this study.

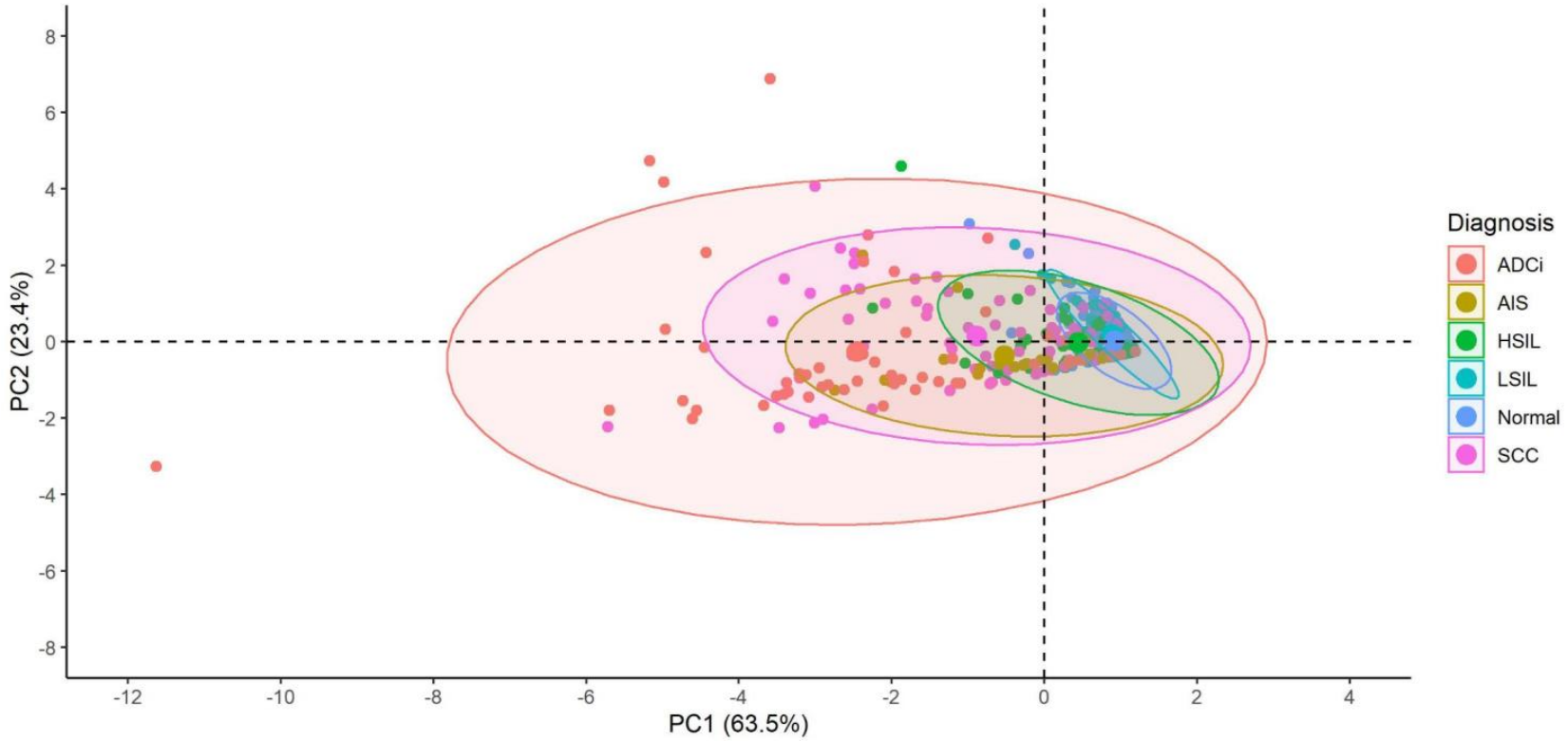
	FFPE [Primer (each):Probe] nM	Cervical Smear [Primer (each):Probe] nM
mQMSP #1		
β-ACT	[200:100]	[200:200]
CADM1	[200:50]	[200:200]
MAL	[200:50]	[400:200]
mQMSP #2		
FAM19A4	[200:50]	[200:200]
hsa-miR-124-2	[200:50]	[400:200]

Supplementary Table 4 – Performance of promoter gene methylation as biomarkers in cervical smears for detection of histological diagnosis of HSIL or worse using Multiple ROC Curve Analysis

Gene	Specificity % (CI[a] 95%)	Sensitivity % (CI 95%)	PPV[b] % (CI 95%)	NPV[c] % (CI 95%)	Accuracy (CI 95%)
FAM19A4/MAL^{me}	51.68 (22.81-80.55)	75.52 (59.99-91.05)	68.66 (54.18-83.14)	79.14 (63.64-94.63)	47.42 (19.78-75.06)
hsa-miR124-2^{me}/MAL^{me}	56.24 (27.61-84.86)	67.56 (50.64-84.47)	64.31 (49.35-79.28)	79.02 (62.57-95.47)	42.65 (17.66-67.64)
FAM194^{me}/has-miR124-2^{me}	53.18 (24.28-82.08)	65.65 (48.47-82.83)	62.07 (46.91-77.22)	77.25 (60.20 - 94.31)	39.85 (15.30-64.39)
All 3-genes	59.26 (30.85-87.66)	61.94 (44.34-79.53)	61.22 (46.02-76.42)	78.59 (61.26-95.92)	39.79 (16.49-63.10)

[a] CI – Confident Interval; [b]PPV – Positive Predictive Value; [e]NPV – Negative Predictive Value

Supplementary Figure 1 – Principal Component Analysis of formalin-fixed paraffin embedded (FFPE) samples group according to histological subtype included in this study. Scatterplot of samples according with methylation levels.



CHAPTER VI –
Research Paper IV

Research Paper IV

Paper IV – Methylation-specific droplet digital PCR: testing a novel triage tool for HrHPV-positive women in the Cervical Cancer Screening Program of Northern Portugal

In preparation

Sofia Salta, José Pedro Sequeira, Ana Teixeira-Marques, Bárbara Amorim-Fernandes, Diana Inês Machado, Sara Saraniero, João Lobo, Ana Sousa, Hugo Sousa, Inês Baldaque, Paula Monteiro, Fernando Tavares, Valérie Taly, Rui Henrique[#], Carmen Jerónimo[#]

Author's personal contribution:

Participation in study design, sample preparation for molecular analyses, namely bisulfite treatment, ddMSP optimization and performance, data analysis, and manuscript drafting.

Methylation-specific droplet digital PCR: testing a novel triage tool for HrHPV-positive women in the Cervical Cancer Screening Program of Northern Portugal

Sofia Salta[1,2], José Pedro Sequeira[1,3,4], Ana Teixeira-Marques[1], Bárbara Amorim-Fernandes[1], Diana Inês Machado[1], Sara Saraniero[1], João Lobo[1,5,6], Ana Sousa[7,8], Hugo Sousa[7,9], Inês Baldaque[9], Paula Monteiro[5], Fernando Tavares[10], Valérie Taly[11], Rui Henrique [1,5,6#], Carmen Jerónimo[1,6#]

[1]Cancer Biology & Epigenetics Group, Research Center of IPO Porto (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), R. Dr. António Bernardino de Almeida, 4200-072, Porto, Portugal

[2]Doctoral Program in Molecular Pathology and Genetics, ICBAS – School of Medicine and Biomedical Sciences – University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-513, Porto, Portugal

[3]Doctoral Program in Biomedical Sciences, ICBAS – School of Medicine and Biomedical Sciences – University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-513, Porto, Portugal

[4]Epigenomics Unit, Cancer Epigenomics, Translational Medical Oncology Group (ONCOMET), Health Research Institute of Santiago de Compostela (IDIS), University Clinical Hospital of Santiago (CHUS/SERGAS), Santiago de Compostela, Spain.

[5]Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), R. Dr. António Bernardino de Almeida, 4200-072, Porto, Portugal

[6]Department of Pathology and Molecular Immunology, ICBAS – School of Medicine and Biomedical Sciences – University of Porto, Rua Jorge Viterbo Ferreira 228, 4050-513, Porto, Portugal

[7]Molecular Oncology and Viral Pathology Group, Research Center of IPO Porto (CI-IPOP) / RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), R. Dr. António Bernardino de Almeida, 4200-072, Porto, Portugal

[8]Escola Superior de Saúde Fernando Pessoa, R. Delfim Maia 334, 4200-256 Porto, Portugal

[9]Department of Pathology and Laboratory Medicine, Clinical Pathology Service, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center Raquel Seruca (Porto.CCC), R. Dr. António Bernardino de Almeida, 4200-072, Porto, Portugal

[10]Administração Regional de Saúde do Norte, I.P., Rua de Santa Catarina 1288, 4000-477, Porto, Portugal.
[11]Centre de recherche des cordeliers, Équipe 26, Équipe labellisée ligue nationale contre le cancer, UMR S1138, CNRS SNC 5096, 15 rue de l'école de médecine, 75006 Paris

#Joint Senior and Corresponding Authors:
Portuguese Oncology Institute of Porto (IPO Porto), Rua Dr António Bernardino de Almeida; 4200-072 Porto, Portugal; Tel: +351 225084000; Fax: + 351 225084047
Email: henrique@ipoporto.min-saude.pt / carmenjeronimo@ipoporto.min-saude.pt
Twitter: @GEBC_IPOPorto

Keywords: Cervical Cancer Screening; DNA methylation; Triage; Colposcopy referral;

Abstract

Cervical cancer screening remains key for cervical cancer prevention. Over the years, continuous improvements have been carried out in cervical cancer screening programs, with high-risk human papillomavirus (HrHPV) testing becoming gold-standard as primary test. Despite its high negative predictive value (NPV), specificity is suboptimal, entailing the need for more effective triage biomarkers to manage HrHPV-positive women, namely those based on DNA methylation. Herein, we sought to develop a droplet digital methylation-specific PCR (ddMSP)-based test for assessing DNA methylation in HrHPV-positive samples and evaluate its performance as triage tool in a population-based cervical cancer screening program.

Firstly, *MAL* and *hsa-miR124-2* methylation levels were assessed by ddMSP in an exploratory setting of 62 HrHPV-positive cervical scrapes. Then, a consecutive series of 750 HrHPV-positive cervical scrapes was used as replication series.

We found significantly higher MAL^{me} and $miR124\text{-}2^{me}$ levels in high-grade squamous intraepithelial lesions or worse (HSIL+) in both exploratory and replication series. In the later, the methylation panel disclosed high specificity and NPV (89% for both) but limited sensitivity. However, when combined with HrHPV genotyping (HPV16/HPV33), 70% sensitivity and approximately 80% specificity and accuracy was achieved.

We concluded that this new triage strategy for HrHPV-positive women might reduce the false positive rate and the percentage of women referred to colposcopy. Thus, a ddMSP-based test allied with HrHPV genotyping has the potential to improve the efficiency and sustainability of a population-based cervical cancer screening program.

Introduction

Cervical cancer remains a health problem worldwide. Despite global efforts to eradicate cervical cancer, it ranks 4th in incidence and cancer-related mortality among women, worldwide [1-3]. Cervical cancer screening was the first measure of cancer prevention implemented in many countries, followed in more recent years by vaccination against Human Papillomavirus (HPV). Although the later constitutes an essential primary prevention resource, screening is key to cervical cancer control and elimination[4].

Persistent high-risk HPV (HrHPV) infection is the leading risk factor for cervical cancer development, being virtually present in all cervical cancers [3]. The most recent European guidelines endorsed HrHPV testing as the most cost-effective primary method for cervical cancer screening, replacing cytology-based screening, the gold standard for several decades [5-7]. HrHPV-based screening is highly sensitive with a high negative predictive value (NPV). However, its positive predictive value (PPV) is limited due to detection of transient HrHPV infections, especially among young women [8]. This limitation entails increased rates of referral to colposcopy units as HrHPV-test replaced cytology [8], ultimately leading to overdiagnosis and overtreatment. Thus, several triage methods have been proposed to stratify risk among HrHPV-positive women. The most cost-effective triage strategies recommended include cytology (alone or combined with HPV16/18 genotyping) [9-11]. However, cytology is highly operator-dependent, and its subjectivity increases with the knowledge of a previous HrHPV-positive result [12,13]. Thus, alternative triage methods have emerged, such as dual staining p16/Ki67 (also known as CINtec™ PLUS) and DNA methylation markers [14,15]. DNA methylation is a well-known epigenetic alteration associated with gene regulation and its potential as a source of biomarkers for cervical cancer screening, including detection and triage, has been reported over the last years [16,17]. Several genes and their combination into panels have been assessed. *CADM1, MAL, FAM19A4,* and *hsa-miR124-2* are among the most studied genes, with thousands of samples analyzed reported in the literature [16]. In a previous study, we investigated the potential of these biomarkers for cervical cancer screening in the Portuguese population [18]. We found that promoter methylation levels of *MAL, FAM19A4,* and *hsa-miR124-2* were significantly higher in high-grade squamous intraepithelial lesions (HSIL) or worse (HSIL+) lesions, in both tissue and cervical scrape samples [18]. However, despite the potential disclosed in our work, some technical limitations were pointed out, namely the need of preamplification steps for analysis of cervical scrapes [18]. To overcome this limitation, more sensitive technologies for methylation assessment such as droplet digital PCR (ddPCR) are required. Indeed, ddPCR have been proven to be more robust (it does not need a standard curve for quantification), accurate (especially with low-template input samples) and less biased

than conventional PCR[19]. Moreover, ddPCR was already shown to be an alternative to real-time quantitative PCR (RT-qPCR) for assessment of miRNAs levels, obviating the need for preamplification in low-input samples such as plasma [20,21]. Likewise, droplet digital methylation-specific PCR (ddMSP) has been also reported as a superior alternative to quantitative methylation-specific PCR (QMSP) for assessment of gene methylation status [22-25]. Thus, we sought to develop a ddMSP-based assay to be used as a triage tool for referral to colposcopy of HrHPV-positive women in a population-based cervical cancer screening program.

Material and Methods

Study Population and Sample Selection

This retrospective study was carried out with the leftover samples primarily used for diagnosis. Thus, cervical scrapes from women enrolled in the Regional Cervical Cancer Screening Program of Northern Portugal were selected. All the samples were collected in a methanol-based, buffered preservative solution (ThinPrep™ PreservCyt™), received at the Department of Pathology of Portuguese Oncology Institute of Porto (IPO Porto) and tested for HrHPV, as previously described [26]. Briefly, 14 HrHPV genotypes (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66, and HPV-68) were simultaneously detected using Anyplex II HPV HR Detection kit (Seegene, Seoul, South Korea). For all samples, DNA was extracted and stored. In all HrHPV-positive cases, cytological examination was performed by experienced cytopathologists. According to the current screening program flowchart, women who tested positive for HrHPVnon-HPV16/18 HrHPV and have a cytological result of Atypical Squamous Cell of Undetermined Significance (ASC-US) or worse are referred to colposcopy; women positive for HPV16 or HPV18 are directly referred to colposcopy, regardless of cytological result; women positive for HrHPVnon-HPV16/18 HrHPV and a cytological result of Negative for Intraepithelial Lesion or Malignancy (NILM) repeat screening after one year. All HrHPV-negative cases are invited to repeat the cervical cancer screening after five years.

For this study, two cohorts of patients were selected: exploratory and replication cohorts. Relevant clinical data is depicted in Supplementary Tables S1 and S2.

For the exploratory series, 62 samples were selected from a previous cohort of patients [18] who tested positive for HrHPV and were referred to colposcopy between March and May of 2019. Only patients who had colposcopy information available were selected. Patients who did not attend colposcopy were excluded from this analysis.

For the replication series, sample selection was performed according to Figure 1. All the samples received at the Department of Pathology of IPO Porto in January 2020 that

tested positive for HrHPV were considered eligible, except thosewithout clinical information or enough DNA stored, which were excluded from analysis. This study was approved by the institutional review board of IPO Porto (Comissão de Ética para a Saúde – CES-371/2017).

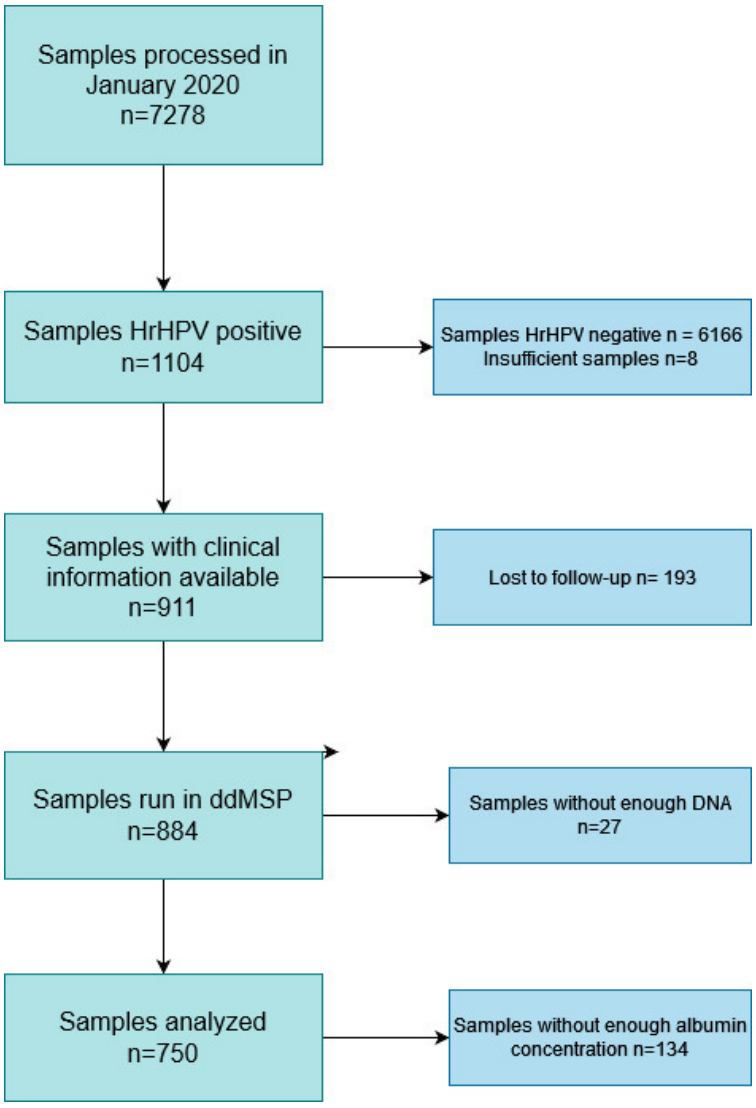


Figure 1 – Flowchart of sample selection for replication setting. Abbreviations: ddMSP – droplet digital Methylation-Specific PCR; HrHPV – high-risk Human Papillomavirus.

Methylation analyses

From all cervical scrapes (exploratory and replication series), we used the leftovers of DNA from the regional screening program extracted in an automated system as previously described [26]. DNA was stored at -80ºC until further use.

Bisulfite treatment was performed for all the samples using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, California), as previously described [18]. For control purposes, Human HCT116 DKO Methylated DNA [positive control (Zymo Research, Orange, California)] and Human HCT116 DKO Non-Methylated DNA [negative control

(Zymo Research, Orange, California)] were quantified using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts), bisulfite-treated, and eluted at the final concentration of 2.5ng/μL.

Further, ddMSP reactions were prepared with 11μL ddPCR Supermix for Probes (No dUTP) (Bio-Rad, Hercules, California), 2μL of bisulfite-treated DNA, primers, and probes according to Supplementary Table S3 and bidistilled water up to final volume 22μL. Droplets were generated using Droplet Generator QX200 (Bio-Rad, Hercules, California). A positive, a negative, and a non-template control were included in each plate. The PCR run was set as follows: 95ºC for 10 minutes, 45 cycles of 94ºC for 15 seconds and, 57.3ºC for 1 minute, 98ºC for 10 minutes. All the steps were run with a 2.5ºC ramp/rate in C1000 Touch (Bio-Rad, Hercules, California). After the PCR reaction, the plate was read on the QX200 Droplet Reader (Bio-Rad, Hercules, California). Plates were analyzed using QX Manager 1.2 Standard Edition (Bio-Rad, Hercules, California). Wells were considered valid if at least 10,000 droplets were read.

The Limit of Blank (LOB) and the Limit of Detection (LOD) were calculated for each target according to the previously described method [27]. Briefly, 30 wells with negative control were run. Methylation ratios for each target gene were calculated by dividing copies/μL of the target by copies/μL of albumin. Samples were considered valid for analysis if albumin concentration was equal to or higher than 15 copies/μL.

Statistical Analysis

Two-group comparisons were performed using the Mann-Whitney U test. To assess the biomarker performance of the methylation markers for HSIL+ detection (according to histological results), Receiver Operator Characteristic (ROC) curves were built, and the area under the curve (AUC) was calculated. Normal (including normal cervix, metaplasia, and cervicitis) and Low-grade Squamous Intraepithelial Lesion (LSIL) samples were considered the control group. The cut-off for each target gene was established at 90% specificity based on the ROC curve in the exploratory series to categorize the samples as methylated or unmethylated. The cut-off established in the exploratory series was applied directly in the replication series. When panels were used, samples were considered positive if at least one parameter was positive.

Furthermore, sensitivity, specificity, PPV, NPV, accuracy, and corresponding 95% confidence intervals (CI) were calculated for HSIL+ detection. The referral rate was calculated as the percentage of women who tested positive for the biomarkers among all women tested. Pre- and Post-test Probabilities were calculated as previously described [28,29]. Two-tailed p-values were derived from statistical tests using IBM SPSS Statistics (Version 27.0, Chicago, Illinois), and a p-value <0.05 was considered

statistically significant. Graphs were assembled using GraphPad 8 Prism (GraphPad Software, Boston, Massachusetts).

Results

LOB and LOD

The LOB was set at one droplet and two droplets for *hsa-miR124-2* and *MAL* methylation, respectively. The LOD was five and six droplets for *hsa-miR124-2* and *MAL* methylation, respectively.

Methylation levels of the *hsa-miR124-2* and *MAL* in the exploratory series

The exploratory cohort comprised 62 women who tested HrHPV-positive with a median age of 41 years old (range: 24 to 61 years).

Methylation levels were assessed in cervical scrapes using ddMSP. All 62 samples were considered valid for analysis. *MAL* and *hsa-miR124-2* promoter methylation levels significantly differed between HSIL+ and Normal & LSIL groups (*p-value*=0.0013 and *p-value*=0.0344, respectively) (Supplementary Figure S1). To maximize specificity, the cut-off was set at 0.01142 for *hsa-miR124-2^{me}* and at 0.00034 for *MAL^{me}*. Thus, 88.89% (CI 95%: 70.2-98-07) and 91.11% (CI 95%: 82.80-99.43) specificity for *hsa-miR124-2^{me}* and *MAL^{me}*, respectively, was achieved (Table 1). When both markers were combined, 80.00% specificity achieved, with limited sensitivity (47.06%) and about 70.00% accuracy above.

Table 1 – Biomarker performance of promoter methylation for detection of high-grade intraepithelial lesion or worse in the exploratory series.

Biomarker	Normal & LSIL n= 45 n (%)[1]	HSIL + n=17 n (%)[1]	Specificity % (CI 95%)	Sensitivity % (CI 95%)	PPV % (CI 95%)	NPV % (CI 95%)	Accuracy (CI 95%)
hsa-miR124-2^{me}	5 (11.11)	5 (29.41)	88.89 (79.71-98.07)	29.41 (7.75-51.07)	50.00 (19.01-80.99)	76.92 (64.61-89.23)	72.58 (61.48-83.69)
MAL^{me}	4 (8.89)	7 (41.18)	91.11 (82.80-99.43)	41.18 (17.78-64.57)	63.64 (35.21-92.06)	80.39 (68.79-91.99)	77.42 (67.01-87.83)
hsa-miR124-2^{me} /MAL^{me}	9 (20.00)	8 (47.06)	80.00 (68.31-91.69)	47.06 (23.33-70.79)	47.06 (23.33-70.79)	80.00 (68.31-91.69)	70.97 (59.67-82.27)

[1] Number of positives cases and percentage of the total; Abbreviations: CI – Confidence interval; HSIL+ – High-grade Squamous Intraepithelial Lesions or worse; LSIL - Low-grade Squamous Intraepithelial Lesions; n – number; NPV – Negative Predictive Value; PPV – Positive Predictive Value.

Methylation as triage for referral to colposcopy of HrHPV-positive women

Methylation levels in cervical scrapes from women participating in the Northern Portugal Cervical Cancer Screening Program that tested positive for HrHPV were evaluated. All

HrHPV-positive samples with DNA stored available were selected for the replication series, as depicted in Figure 1.

From 884 samples with DNA available for bisulfite treatment and run in ddMSP, 750 were considered valid for analysis. The cohort comprised HrHPV positive women with a median age of 39 years old (range: 25 to 64 years).

Promoter methylation levels remained statistically different between HSIL+ and Normal & LSIL groups [*p-value*=0.0008 and *p-value*<0.0001, for *hsa-miR124-2^{me}* and *MAL^{me}*, respectively (Supplementary Figure S2)].

Using the positivity cut-off established in the exploratory series, high specificity (above 90%) was achieved for each target gene individually. Moreover, accuracy and NPV were above 83% for *hsa-miR124-2^{me}* and *MAL^{me}.* When both markers were combined, there was a slight increase in sensitivity without relevant change in specificity or accuracy (Table 2).

Table 2 – Biomarker performance of promoter methylation and HPV genotyping for detection of high-grade intraepithelial lesion or worse in the replication series.

Triage algorithms	Normal & LSIL n= 658 n (%)[1]	HSIL + n=92 n (%)[1]	Specificity % (CI 95%)	Sensitivity % (CI 95%)	PPV % (CI 95%)	NPV % (CI 95%)	Accuracy (CI 95%)
hsa-miR124-2^{me}	43 (6.53)	16 (17.39)	93.47 (91.58-95.35)	17.39 (9.65-25.14)	27.12 (15.77-38.46)	89.00 (86.61-91.39)	84.13 (81.52-86.75)
MAL^{me}	35 (5.32)	17 (18.48)	94.68 (92.97-96.40)	18.48 (10.55-26.41)	32.69 (19.94-45.44)	89.26 (86.89-91.62)	85.33 (82.80-87.87)
hsa-miR124-2^{me} /MAL^{me}	71 (10.79)	23 (25.00)	89.21 (86.84-91.58)	25.00 (16.15-33.85)	24.47 (15.78-33.16)	89.48 (87.14-91.83)	81.33 (78.54-84.12)
***hsa-miR124-2^{me} /MAL^{me}*+ HPV16/18/33**	151 (22.94)	64 (69.57)	77.05 (73.84-80.26)	69.57 (60.16-78.97)	29.77 (23.66-35.88)	94.77 (93.06-96.47)	76.13 (73.08-79.18)
***hsa-miR124-2^{me} /MAL^{me}*+ HPV16/33**	135 (20.52)	63 (68.48)	79.48 (76.40-82.57)	68.48 (58.98-77.97)	31.82 (25.33-38.31)	94.75 (93.04-96.45)	78.13 (75.18-81.09)
HPV 16/18 + Cytology (current)	226 (34.34)	72 (78.26)	65.65 (62.03-69.28)	78.26 (69.83-86.69)	24.16 (19.30-29.02)	95.58 (94.00-97.15)	67.20 (63.84-70.56)

[1] Number of positives cases and percentage of the total; **Abbreviations:** CI – Confidence interval; HPV – Human Papillomavirus; HSIL+ – High-grade Squamous Intraepithelial Lesions or worse; LSIL - Low-grade Squamous Intraepithelial Lesions; n – number; NPV – Negative Predictive Value; PPV – Positive Predictive Value.

Despite superior specificity and accuracy, methylation markers disclosed limited sensitivity. Thus, HPV genotyping information was added to the methylation markers. We combined the methylation markers with HPV-16, HPV-18, and HPV-33 due to the higher risk of HSIL+ in women positive for these HrHPV genotypes [30,31]. Combined with partial HPV genotyping, methylation markers achieved a sensitivity of nearly 70%, with a specificity and accuracy above 75% (Table 2). Removing HPV-18, since its prevalence among the Portuguese HrHPV positive women is nearly 4% [31], results in a slight increase in specificity and accuracy but at the cost of decreased sensitivity.

The current used strategy (HPV16/18 + cytology) disclosed 65.65% (CI 95%: 62.03-69.28) specificity, with similar accuracy [67.20 % (CI 95%:63.84-70.56)] and higher sensitivity [78.26% (CI 95%: 69.83-86.69)].

Moreover, we assessed the percentage of women sent to colposcopy and the false positive rate for the current strategy and the proposed new strategies. Both methylation + HrHPV genotyping displayed a lower false positive rate, and percentage of women sent to colposcopy with higher accuracy (Table 3).

Table 3 – Referral to colposcopy and false positive rates for proposed and current strategies of triage for HrHPV-positive women

Triage algorithms	Accuracy (CI 95%)	False positive rate (CI 95%)	Referral rate (%)
***hsa-miR124-2^{me} /MAL^{me}*+ HPV16/18/33**	76.13 (73.08-79.18)	22.95 (19.74-26.16)	28.7
***hsa-miR124-2^{me} /MAL^{me}*+ HPV16/33**	78.13 (75.18-81.09)	20.52 (17.43-23.60)	26.4
HPV 16/18 + Cytology (current)	67.20 (63.84-70.56)	34.35 (30.72-37.97)	39.7

Abbreviations: CI – Confidence interval; HPV – Human Papillomavirus

A double positive result with a new strategy of triage (HPV16/33+methylation) was associated with a higher probability of HSIL+ than the current strategy (HPV16/18+reflex cytology) [66% *vs.* 60% (Figure 2 and Supplementary Table S4)].

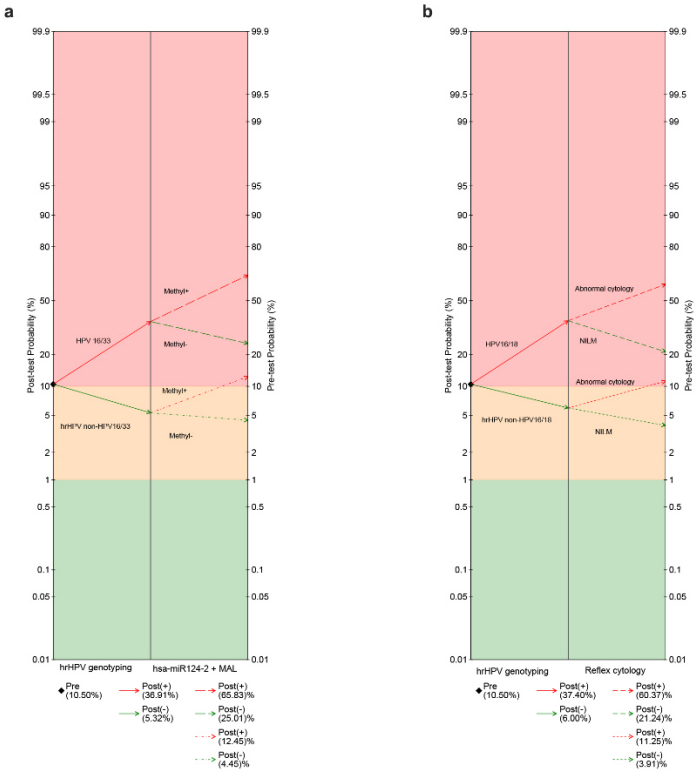


Figure 2 – Pre-test and post-test probability (%) of high grade squamous intraepithelial lesion (HSIL) or worse for the newly proposed strategy [HrHPV genotyping (HPV16/33) and *hsa-miR124-2^{me}*/MAL^{me} (a)] and for current strategy

[HrHPV genotyping (HPV16/HPV18) and reflex cytology (b)]. Abbreviations: HPV – Human Papillomavirus; HrHPV – high risk HPV; Methyl+ – positive for at least one methylation marker; Methyl- – negative for both methylation markers; NILM – Negative for Intraepithelial Lesion or Malignancy.

Discussion

Screening remains key to cervical cancer control and an important strategy to achieve cervical cancer elimination, as proposed by the World Health Organization (WHO) [3]. In recent years, HrHPV testing became gold standard for cervical cancer screening in developed countries, following new international guidelines [32,33]. In the Cervical Cancer Screening Program of Northern Portugal, established in 2009, using cytology as primary test, the implementation of first line HrHPV testing in 2017 led to increased referral to Cervical Pathology Units, increasing the waiting time for observation and treatment due to the overload of colposcopy units [34]. Despite its substantial NPV, HrHPV testing has limited PPV, entailing overdiagnosis and overtreatment, negatively impacting the sustainability of the program. In this setting, methylation markers have emerged as alternative tool for triaging HrHPV-positive women referred to colposcopy [16], including in the Portuguese population [18].

To the best of our knowledge, this is the first study using ddMSP to assess methylation status in cervical scrapes. This technology was reported as more accurate and less biased than RT-qPCR, including in liquid biopsies [24,25]. Moreover, we have previously demonstrated that ddPCR avoids preamplification in low input samples and better replicates the tumor burden than RT-qPCR [21]. Indeed, using ddMSP instead of qMSP allowed for overcoming the need for the preamplification step which has been associated with increased bias and false positive rate [35].

Despite good accuracy, specificity, and NPV, methylation markers alone disclosed limited sensitivity. The storage time of low-concentrated samples allied to bisulfite treatment might negatively influence our results. Nonetheless, when methylation biomarkers were combined with HPV genotyping, performance increased with nearly 70% sensitivity and approximately 80% specificity and accuracy. The performance of this algorithm emulates or even surpasses that of most host-gene methylation-based markers reported in the literature for HSIL+ detection in HrHPV-positive women [16,36]. Interestingly, methylation markers have been previously combined with partial HrHPV genotyping [37-39], with a double negative result associated with a reduction in post-test probability of cervical intraepithelial neoplasia (CIN) 3 or worse lesions (CIN3+), similar to our results.

Dick and colleagues have shown that a negative *FAM19A4*/*miR124-2* methylation result and HPV16/18 was associated with a long-term (14 years follow-up) low CIN3+ risk (10.7%) [37]. Additionally, the same researchers demonstrated that negative methylation results for the same genes were associated with a high regression rate of HSIL. In that

interesting study, women with biopsy-confirmed CIN2 or CIN3 were monitored every six months for two years to look for progression or regression. Remarkably, about ¾ of the women in this study were young (under 35 years old) in which fertility preservation and reduction of the risk of obstetric complications is of paramount importance [40]. Moreover, a similar performance in risk stratification was verified in both self-collected and clinician-collected samples [40]. Thus, methylation markers are a promising tool to triage HrHPV-positive women in self-collected samples [41-44], a methodology which is increasingly adopted by screening programs.

Likewise, Verhoef et al. reported the potential of *MAL* and *miR124-2* methylation for triage of HrHPV-positive women in self-collected samples [43,44]. The PROHTECT-3 trial reported an unadjusted sensitivity for HSIL+ detection of 72.6% but with limited specificity (42.61%, unadjusted) [43]. However, when several specificity cut-offs were tested, using threshold-80 (standing for 80% of specificity for CIN3+ detection) the specificity for CIN2 or worse (CIN2+) detection rose to 83% with a decrease of sensitivity to 33.8% [44]. When combined with HPV16/18 genotyping, the authors reported 55% specificity and 67.1% sensitivity [44]. We defined 90% specificity for HSIL+ detection for defining the methylation cut-off, leading to limited sensitivity. However, addition of HPV16/33 genotyping to the triage algorithm increased the sensitivity to nearly the values reported in the literature, with higher specificity [16,43,44]. HPV 16/18 genotyping as triage of HrHPV-positive women has proven to be cost-effective with or without association with cytology in non-HPV16/18 HrHPV-positive women [10,11,45]. Nevertheless, extended genotyping has emerged as an important tool to assess CIN3+ risk [46]. Indeed, HPV33 was reported as the second HrHPV genotype (after HPV-16) with a higher risk for CIN3+, including in the Portuguese population [30,31,46,47]. Overall, the algorithm we propose increases the efficiency of the cervical cancer screening program by reducing the proportion of women referred to colposcopy in more than 10%, compared to the currently used strategy.

In conclusion, our findings disclose a novel strategy to improve the triage for colposcopy of HrHPV-positive women. Although future validations using prospective samples and management of HrHPV-positive and triage-negative women are still required, this strategy has the potential to increase the cost-effectiveness of the regional screening program, providing a faster and more reliable referral pathway to the women enrolled.

Declarations

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Authors' Contributions

SoS prepared samples for molecular analyses, bisulfite treatment, performed ddMSP optimization and execution, analyzed data, and drafted the manuscript. JPS assisted in ddMSP optimization, sample preparation (bisulfite treatment) and data analysis. AT-M, BA-M, DC-M and SaS assisted in sample preparation (bisulfite treatment and ddMSP assay). AS and HS performed DNA extraction from cervical scrapes. VT assisted in ddMSP optimization and design. PM and IB supervised all the samples selection, DNA extraction, and data acquisition from the Cervical Cancer Screening Program of Northern Portugal, with the assistance of FT. JL collected clinical follow-up data from Northern Cervical Cancer Screening program samples. RH and CJ designed and supervised the study and revised the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the institutional review board of Portuguese Oncology Institute of Porto (Comissão de Ética para a Saúde – CES-371/2017).

Consent for publication

Not applicable

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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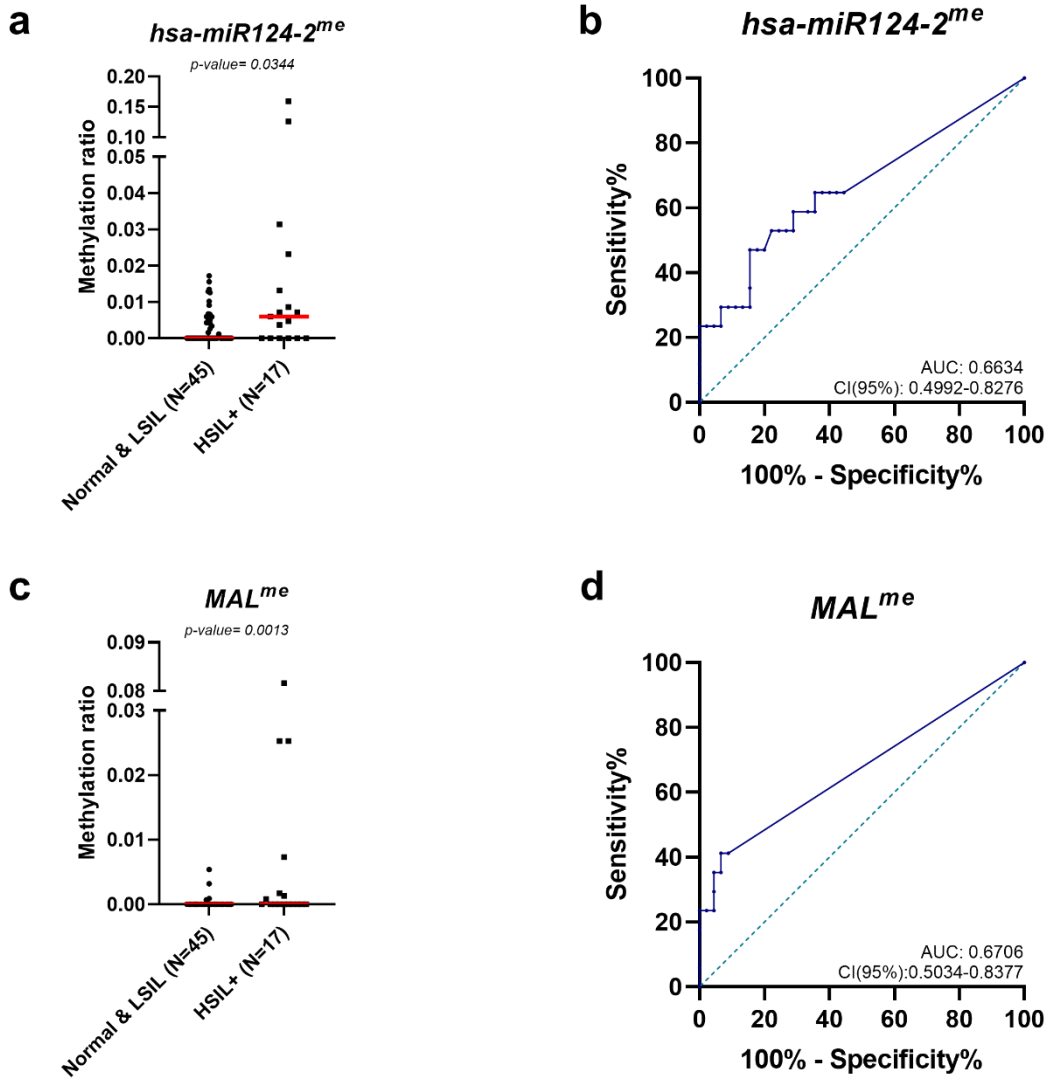
Supplementary Material

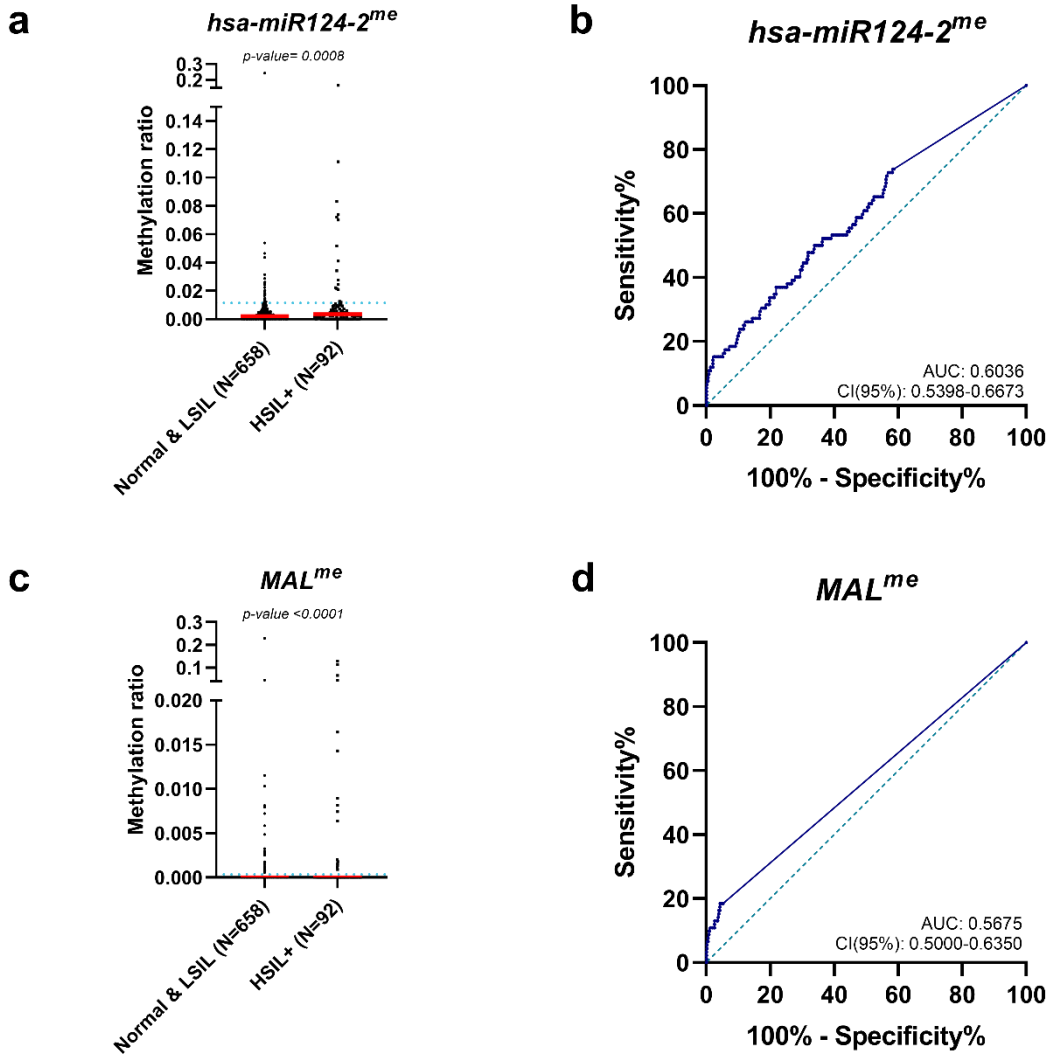
Salta et al. Supplementary Figures – Methylation-specific droplet digital PCR: a tool to triage HrHPV-positive women in Cervical Cancer Screening Program of Northern Portugal

Supplementary Figure S1



Supplementary Figure S1 – Scatter plots (a,c) and Receiver Operating Characteristic (ROC) Curve (b,c) for *hsa-miR124-2* (upper panel) and *MAL* (lower panel) promoter's methylation level in exploratory series. Red lines depict median methylation levels.

Supplementary Figure S2



Supplementary Figure 2 – Scatter plots (a,c) and Receiver Operating Characteristic (ROC) Curve (b,c) for *hsa-miR124-2* (upper panel) and *MAL* (lower panel) promoter's methylation level in replication series. Red lines depict median methylation levels. Blue dot lines in scatter plots represent the cut-off stablished in exploratory series.

Salta et al. Supplementary Tables – Methylation-specific droplet digital PCR: a tool to triage HrHPV-positive women in Cervical Cancer Screening Program of Northern Portugal

Supplementary Table S1 – Clinicopathological features of women of exploratory cohort.

Clinical features	n=62
Age [median (range)]	41 (24-61)
HPV result [n, (%)][1]	
HPV-16	9 (14.5%)
HPV-18	2 (3.2%)
HPV-31	4 (6.5%)
HPV-33	2 (3.2%)
HPV-35	1 (1.6%)
HPV-39	1 (1.6%)
HPV-45	1 (1.6%)
HPV-51	1 (1.6%)
HPV-52	2 (3.2%)
HPV-56	3 (4.8%)
HPV-58	2 (3.2%)
HPV-59	1 (1.6%)
HPV-66	4 (6.5%)
HPV-68	3 (4.8%)
Multiple infections with HPV-16/18	9 (14.5%)
Multiple infections without HPV-16/18	17 (27.4%)
Cytological result [n, (%)]	
NILM	6 (9.7%)
ASC-US	32 (51.6%)
LSIL	12 (19.4%)
ASC-H	7 (11.3%)
HSIL	3 (4.8%)
AGC	1 (1.6%)
Insufficient/unsatisfactory	1 (1.6%)
Histological result [n, (%)]	
No lesion[2]	16 (24.2%)
LSIL	14 (22.6%)
HSIL	17 (27.4%)
Not available	15 (24.2%)

[1] – The sum of each HrHPV genotype cases percentage is higher than 100% due to the existence of cases with multiple HrHPV infections; [2] In this group are included cervicitis, metaplasia and the normal histology. **Abbreviations:** AGC – Atypical Glandular Cells; ASC-H – Atypical Squamous Cells - cannot exclude High grade squamous intraepithelial lesion; ASC-US – Atypical Squamous Cells of Undetermined Significance; HSIL – High-grade Squamous intraepithelial lesions; LSIL – Low-grade Squamous intraepithelial lesions; n – number; NILM – Negative for Intraepithelial Lesion Malignancy.

Supplementary Table S2 – Clinicopathological features of women of replication cohort.

Clinical features	n=750
Age [median (range)]	39 (25-64)
HPV result [n, (%)][1]	
HPV-16	53 (7.1%)
HPV-18	10 (1.3%)
HPV-31	66 (8.8%)
HPV-33	18 (2.4%)
HPV-35	19 (2.5%)
HPV-39	40 (5.3%)
HPV-45	16 (2.1%)
HPV-51	50 (6.7%)
HPV-52	58 (7.7%)
HPV-56	44 (5.9%)
HPV-58	31 (4.1%)
HPV-59	22 (2.9%)
HPV-66	49 (6.5%)
HPV-68	63 (8.4%)
Multiple infections with HPV-16/18	54 (7.2%)
Multiple infections without HPV-16/18	157 (20.9%)
Cytological result [n, (%)]	
NILM	513 (68.4%)
ASC-US	138 (18.4%)
LSIL	53 (7.1%)
ASC-H	30 (4.0%)
HSIL	12 (1.6%)
Insufficient/unsatisfactory	4 (0.5%)
Histological result [n, (%)]	
No lesion[2]	85 (11.3%)
LSIL	179 (23.9%)
HSIL	90 (12.0%)
SCC/AIS	2 (0.3%)
Not available/ not applicable	394(52.5%)

[1] – The sum of each HrHPV genotype cases percentage is higher than 100% due to the existence of cases with multiple HrHPV infections; [2] In this group are included cervicitis, metaplasia and the normal histology. **Abbreviations:** AIS – Adenocarcinoma *in situ*; ASC-H – Atypical Squamous Cells - cannot exclude High grade squamous intraepithelial lesion; ASC-US – Atypical Squamous Cells of Undetermined Significance; HSIL – High-grade Squamous intraepithelial lesions; LSIL – Low-grade Squamous intraepithelial lesions; n – number; NILM – Negative for Intraepithelial Lesion Malignancy; SSC – Squamous cell carcinoma.

Supplementary Table S3 –Primers and Probes sequences and concentrations in PCR reactions used in this study.

Gene	Sequence (5'-3')	Concentration [nM]
hsa-miR124-2	F: GGTAATTAATTTGGATTTACGTCGTTAT	400
	R: GTAAAAATATAAACGATACGTATACCTACG	400
	Probe: 6-FAM/TCTCCATAC/ZEN/CATTTACAACACACG CCTAAA /IABkFQ	250
MAL	F: GCGTAGTATTAAGTAGAGAGGTTCG	400
	R: AATAAAAAATAAAACCGACCGC	400
	Probe: 6-FAM/ACTAAACCG/ZEN/ACGCTAATTCGA CGCT/IABkFQ	125
	Probe: HEX/ACTAAACCG/ZEN/ACGCTA ATTCGACGC T/IABkFQ/	125
Albumin	F: GGGATGGAAAGAATTTTATGTT	400
	R: AAACAAACTAACCCCAAATTCT	400
	Probe: HEX/AGGGTTTTTATAATTTA/MGB-Eclipse	600

Abbreviations: F – primer forward; nM – nanomolar; R – reverse primer

Supplementary Table S4 – Number of women, pre-test and post-test probability

	Women (n)	HSIL or worse (n)	Pre-test probability	Post-test probability
Proposed strategy (*hsa-miR124-2^{me}/MAL^{me}* + HPV16/33)				
HPV16/33	129	53	0.105	0.369
Methylation positive	25	13	0.369	0.658
Methylation negative	104	40	0.369	0.250
Non-HPV16/33	621	39	0.105	0.053
Methylation positive	69	10	0.053	0.125
Methylation negative	552	29	0.053	0.045
Current strategy (HPV16/18 + reflex cytology)				
HPV 16/18	113	47	0.105	0.374
Abnormal Cytology	51	34	0.374	0.604
NILM	61	13	0.374	0.212
non-HPV16/18	452	45	0.105	0.060
Abnormal Cytology	182	24	0.060	0.113
NILM	452	21	0.060	0.039

Abbreviations: HPV – Human Papillomavirus; HSIL – High-grade Squamous Intraepithelial Lesion; n – number; NILM – Negative for Intraepithelial Lesion or Malignancy.

CHAPTER VII –
Major Findings

Major findings

Paper I – Preliminary outcomes of the Cervical Cancer Screening Program of Northern Portugal: a snapshot

- The current flowchart of the Cervical Cancer Screening Program of Northern Portugal follows the best practices reported in the literature.
- HPV16/18 genotyping is associated with higher risk of HSIL+ than abnormal cytology or persistent HrHPV infection.
- Although HrHPV testing has been associated with increased sensitivity for HSIL+ detection, a sizeable proportion of women referred to Cervical Pathology Units did not disclose any lesion during the follow-up period, and some of them effectively cleared the HrHPV infection.
- Continuous improvement in cervical cancer screening programs is essential to ensure sustainability.

Paper II – DNA methylation as a triage marker for colposcopy referral in HPV-based cervical cancer screening: a systematic review and meta-analysis (doi: 10.1186/s13148-023-01537-2)

- Several methylation markers constitute a potential tool for triaging HrHPV-positive women.
- Pooled sensitivity and specificity among all studies were 68% and 75% for CIN2+ detection and 78% and 74% for CIN3+ detection, respectively.
- Promoter methylation of *CADM1, FAM19A4, MAL,* and *hsa-miR124-2* were the most studied biomarkers in this setting, with more than 6500 samples analyzed.
- *CADM1, FAM19A4, MAL,* and *hsa-miR124-2* had a pooled sensitivity of 64%, with pooled specificity of 74% for CIN2+ detection, and pooled sensitivity and specificity of 74% for CIN3+ detection.
- Considering population-based studies, pooled sensitivity and specificity for CIN2+ detection were 60% and 70%, respectively, and 69% and 71%, respectively, for CIN3+ detection.
- Methylation markers disclose slightly inferior performance in self-collected samples than in health professional-collected samples.

- Standardization and validation are still required before methylation biomarkers might be endorsed in clinical guidelines for cervical cancer screening.

Paper III – Performance of DNA methylation-based biomarkers in the cervical cancer program of northern Portugal: A feasibility study (doi: 10.1002/ijc.33778)

- *MAL, FAM19A4* and *hsa-miR124-2* disclosed significantly higher methylation levels in HSIL+ group than Normal & LSIL group in FFPE tissues.
- Individually, in tissues, these markers depicted high specificity (superior to 85%).
- In cervical scrapes from hrHPV-positive women, *MAL, FAM19A4* and *hsa-miR124-2* promoter methylation levels remained significantly higher in HSIL+.
- MAL^{me}, $FAM19A4^{me,}$ and $hsa\text{-}miR124\text{-}2^{me}$ individually reached specificity above 80% with high accuracy (above 70%).
- The 3-markers panel retained high specificity (74%) with 60% sensitivity and 70% accuracy.
- Methylation markers might constitute an important tool for triage of hrHPV-positive women, increasing cervical cancer screening sustainability.

Paper IV – Methylation-specific droplet digital PCR: testing a novel triage tool for hrHPV-positive women in the Cervical Cancer Screening Program of Northern Portugal

- *MAL* and *hsa-miR124-2* displayed significantly higher methylation levels in HSIL+ group.
- In the exploratory setting, MAL^{me} and $hsa\text{-}miR124\text{-}2^{me}$ showed high specificity and accuracy, individually and as a panel.
- In the replication setting (a consecutive series of samples from the screening program), MAL^{me} and $hsa\text{-}miR124\text{-}2^{me}$ retained high specificity (about 89%) and accuracy (above 80%), although with modest sensitivity.
- When combined with HPV genotyping (HPV16/33), specificity and accuracy attained nearly 80% with approximately 70% sensitivity.

- In comparison with the current strategy, applying an algorithm combining gene promoter methylation and HrHPV genotyping decreases the false positive rate by approximately 14% and the referral rate to colposcopy by about 13%.
- This novel algorithm might contribute to increase cervical cancer screening efficiency, cost-effectiveness and sustainability.

CHAPTER VIII –
General Discussion

General Discussion

Cervical cancer remains a major health concern worldwide. Thus, WHO established the 2030 goal of achieving the 90-70-90 strategy: 90% of girls fully vaccinated against HrHPV until 15 years old; 70% of women screened at 35 and 45 years old; 90% of the women with the disease treated [1]. Attaining these goals requires the continuous and sustainable deployment of several initiatives, tackling cervical cancer from several perspectives. One of these is well organized, effective and efficient population-based screening, which constitutes the main theme of this thesis.

In the last decade and owing to cumulative evidence, HrHPV testing has become gold standard for cervical cancer screening due to its superiority in HSIL+ detection compared to cytology [2]. This adjustment in screening strategy came with multiple challenges, including the best triage strategy for HrHPV-positive women due to the high frequency of transient HrHPV infection in the population. Indeed, currently available test are unable to distinguish between persistent or transient HrHPV infection [3]. This testing limitation is associated with an increase in women (inappropriately) referred for colposcopy and, ultimately, leading to overdiagnosis and overtreatment [4].

In line with the gold standard and following European guidelines, the regional Cervical Cancer Screening Program of Northern Portugal, a population-based program fully supported by the Portuguese government, has changed from cytology to primary HrHPV testing. Consequently, the expected increase (estimated as 4-fold) in women´s referral rates for Cervical Pathology Units materialized, causing a significant overload of these units and an increment in waiting times for consultation and eventual treatment [5]. This scenario set the stage for core goal of this thesis: to develop and validate methylation-based markers for a more effective triage of HrHPV-positive women. Therefore, the project was designed to respond to a specific clinical need. Firstly, we addressed the characterization of the current screening strategy to better understand the impact of primary HrHPV test upon the health care system (**Paper I**). Subsequently, we sought to identify potential biomarkers which might improve the efficiency of the screening program (**Paper II**) and further validate them in the Portuguese population (**Paper III**). Finally, using innovative technology (ddMSP), a novel algorithm was developed to better address the challenges of triage of HrHPV-positive women in real-world samples (**Paper IV**).

Thus, the results of this research work, presented in **Chapters III, IV, V,** and **VI,** pinpointed some limitations of the current screening program and unveiled a new approach to improve the regional population-based cervical cancer screening program, namely:

- The cervical cancer program follows high standards for HrHPV-positive women triage, but there is a sizeable percentage of women unnecessarily sent for consultations in Cervical Pathology Units **(Paper I)**.
- Methylation-based markers have shown promise as triage tools in cervical cancer screening programs **(Paper II)**, with three out of the four more studied markers also having potential to be used in the Portuguese population **(Paper III)**.
- The methylation markers combined with HrHPV genotyping represent an alternative to the current triage strategy, decreasing false-positive and referral rates and disclosing high accuracy **(Paper IV).**

Thereby, the most relevant results of this thesis are discussed below.

Impact of HrHPV testing in the screening program

Primary HrHPV testing became the gold standard for many countries worldwide, with an impact on the detection of HSIL+ [2,3]. Nevertheless, in several countries, it was associated with an increase in colposcopy referrals, highlighting the importance of a good triage strategy for HrHPV-positive women [4]. Currently, there is no consensus concerning the best triage strategy in this setting. With this in mind, the first manuscript – **Paper I** – focused on characterizing the current screening strategy: primary HrHPV testing with HPV genotyping and cytology as triage. Although the empirical knowledge makes clear the downsides and benefits of HrHPV primary testing, this is, to the best of our knowledge, the first study which managed to quantitatively characterize the performance of the regional Cervical Cancer Screening Program of Northern Portugal.

A very important finding from this study was the demonstration of the compliance of the screening program with the best practices reported in the literature for triage of HrHPV-positive women. It should be recalled here that cytology remains the most used triage tool, notwithstanding its limitations and flaws. Indeed, its power is limited and influenced by the *a priori* knowledge of the HrHPV test result (imparting a higher likelihood of interpreting cellular alterations as potentially dysplastic) and inter-operator variability [6,7]. Nevertheless, the use of cytology after partial HrHPV genotyping has proven more cost-effective than cytology alone [8-11]. In fact, in our study, the partial genotyping with HPV16/18 was associated with a higher probability of HSIL+ detection. This finding is in line with previous findings on HPV16/18 genotyping as been proven a cost-effective triage tool for screening programs [10,12]. Importantly, partial HPV16/18 genotyping among vaccinated women is associated with high PPV [12,13]. It should be emphasized that, in our study, more than half of the women testing positive for HPV16/18 disclosed a NILM result

in cytology, and among these, about 20% later presented an HSIL+. This clearly demonstrates the importance of HrHPV genotyping and, simultaneously, the added value of screening strategy changing from primary cytology to primary HrHPV testing [14,15].

Nonetheless, about 22% of women sent to a Cervical Pathology Unit in our study effectively cleared the viral infection spontaneously, and nearly 14% underwent a histology biopsy, in which the result was negative for intraepithelial lesions or cancer. This highlights one of the potential harms of HrHPV primary testing, i.e., unnecessary invasive procedures with consequent overdiagnosis, and overtreatment, which might increase the risks of obstetric complications and anxiety among women who tested positive in the screening program [2]. Moreover, an increased false positive rate might reduce screening attendance, increasing the number of women lost to follow-up and the cost associated with the screening program [2].

Thus, alternative biomarkers enabling a more effective triage of HrHPV-positive women have become a clinical need.

DNA methylation as an alternative to cytology triage?

Dual stain p16/Ki67 and methylation markers have been considered the most promising tools to replace cytology as triage test for HrHPV-positive women. Although the dual-stain test overcomes the intra- and inter-observer subjectivity associated with cytology [16], it still requires a high quality specimen of LBC, collected by a health professional. Additionally, its specificity as a single triage strategy is comparable to cytology, although with higher sensitivity [17].

Methylation markers have also become the focus of multiple research projects for detection of HSIL+ and/or triage of HrHPV-positive women, especially considering the growing adoption of self-sampling. In line with this, we aimed to i) systematically collect the candidate methylated genes devised for triage and ii) assess their potential as biomarkers (**Paper II**). In this systematic review and meta-analysis, we were able to demonstrate that multiple methylation markers and panels were reported in the literature for triaging HrHPV-positive women for referral to colposcopy, although high heterogeneity among studies was disclosed. Compared to cytology (having ASC-US+ as threshold) methylation markers showed similar pool specificity for CIN2+ detection but higher for CIN3+ detection [18]. Furthermore, methylation markers demonstrated potential for efficient use in self-collected samples, in which cytology is not an option. In this study we also verified that the most studied methylated gene promoters in a triage setting were *CADM1, FAM19A4, MAL*, and *hsa-miR124-2*, and, therefore, these were selected to be tested in samples obtained from the regional screening program. In this respect, it should

be emphasized that the potential of those markers was essentially demonstrated in the Dutch population. Thus, additional validation in the Portuguese population is of great importance considering the lack of reliable information concerning variations in gene promoter methylation levels across different populations.

Feasibility of using methylation markers in a cervical cancer screening program targeting a Portuguese population

As stated above, despite the potential of methylation markers, validation of their performance in a southern European (Portuguese) population was felt mandatory (**Paper III**). In this study, we demonstrated that *FAM19A4, MAL,* and *hsa-miR124-2* were differentially methylation in the HSIL+ group compared to the Normal & LSIL group. This finding was not restricted to FFPE tissue samples (initially used to assess the specificity and sensitivity of the methylation biomarkers in samples with abundant genomic DNA available) but also to cervical scrapes collected in ThinPrep™ PresevertCyt™. Nevertheless, to attain a clinically meaningful performance, we were faced with the need to carry out preamplification of DNA from cervical scrapes. Although the inclusion of a preamplification step in the protocol does improve the sensitivity of the test, it has several downsides, including a reduction in specificity. Thus, we sought to develop an alternative strategy which might overcome the need for preamplification. With that purpose in mind, we focused our attention in an innovative technology – ddPCR – which enables high sensitivity and subsequently tested it in samples from the regional screening program (**Paper IV**).

The option for ddPCR stemmed from reports demonstrating its superior cost-effectiveness compared to qPCR, since it obviates the need for preamplification in low-input samples, and better represents tumor burden [19,20]. Consequently, it is also less biased and is associated with lower false-positive rates for unspecific amplifications [21]. Indeed, the use of ddPCR for HPV detection has been proven to be more sensitive than qPCR [20] and also as a potential tool for therapy monitoring in cervical and vulvar cancer [22].

The panel which we developed for ddMSP comprised only two genes (*MAL* and *hsa-miR124-2),* which might negatively impact the sensitivity for HSIL+ detection. Thus, we decided to make use of genotyping information to improves the performance of the algorithm, and compare it with the triage strategy current applied in the Cervical Cancer Screening Program in Northern Portugal. The added value of HrHPV genotyping is

controversial topic in the literature [23,24]. Whereas HPV-16 and HPV-18 have been reported as the most oncogenic and, consequently, the HPV genotypes more frequently present in cervical cancers (about 70%), other genotypes have also been described as associated with an increased risk of CIN3+ development [3]. Indeed, Demarco and colleagues demonstrated that, despite HPV-16 being associated with the highest risk for progression to CIN3+ than the other genotypes, which continually increases over follow-up time, HPV-33 was associated with the second highest risk of progression [23]. Interestingly, similar results were found for the Northern Portuguese population [25], and HPV-33 has been previously recommended for incorporation in HPV16/18 triage strategies [26]. Nevertheless, HPV16/33 positive samples represented only about 15% of HrHPV-positive samples from the screening [25], and complementary strategies such as methylation markers have an added value for the cost-effectiveness of the screening program.

Remarkably, our results (research **Paper IV)** demonstrate that methylation markers combined with HrHPV genotyping are able to reduce the false positive rate by approximately 12-14% and the referral rate to cervical pathology units by roughly 11-13%, compared to the currently used strategy. Although the proposed strategy displayed lower sensitivity for HSIL+ detection than the current strategy, constituting a potential drawback, it has been recently reported that a negative result for methylation markers (*FAM19A4* and *miR124-2*) was associated with clinical regression of untreated HSIL [27]. Moreover, a negative result for *FAM19A4* and *hsa-miR124-2* methylation in a triage test has also been associated with lower long-term risk of CIN3+ incidence [28]. These findings, which are especially relevant for women at reproductive age in which fertility preservation is a major concern, indicate that a negative methylation results does not necessarily signify a “false negative”, at the least from a biological and clinical standpoint. Furthermore, this should prompt a more critical view on the definition of the “meaningful” endpoints for biomarker studies.

Previous studies have shown that DNA methylation markers, either of host or HPV genes, have potential for detection of cervical cancer and its precursor lesions [29]. Furthermore, some methylation panels (*CADM1/MAL* [30], *FAM19A4/miR124-2* [31], and *ASCL1/LHX8* [31]) have shown promise for detection of recurrent disease [30,31]. And the use of methylation markers has also been proposed to help predict the risk of progression of untreated HSIL [32] and aid in the management of pregnant women diagnosed with CIN3 [33]. These findings strengthen the use of methylation markers in the context of screening programs since they might represent an indicator of disease severity and help clinicians in decision making. Remarkably, two methylation-based assays have recently received the *Conformité Européene In Vitro Diagnostic* (CE-IVD) label: Gyntect

™ (evaluates methylation levels of *ASTN1, DLX1, ITGA4, RXFP3, SOX17*, and *ZNF671*), and Precursor M+/QIAsure Methylation Test kit (using *FAM19A4* and *hsa-miR124-2*). Nonetheless, concerns regarding biomarkers' sensitivity still preclude their disseminated use and inclusion in WHO guidelines.

We realize that our findings require further validation and technological development to reach clinical use, but we confident that methylation markers, especially combined with HrHPV genotyping hold the key to perfect the management of HrHPV-positive women, enabling a more efficient triage for referral to colposcopy, increasing the cost-effectiveness and the sustainability of the regional cervical cancer screening program.

References (Chapter VIII)

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CHAPTER IX – Conclusions and Future Perspectives

Conclusions and Future Perspectives

Albeit the main goal of this thesis was to develop a methylation-based markers panel for the triage of HrHPV-positive women to referral for colposcopy, this work disclosed some important features that may lead to a better understanding of the cervical cancer screening program's current reality and enable further improvements, as well as potential solutions.

Unveiling strengths and weaknesses

In **Paper I,** we described the strengths and weaknesses of the current cervical cancer screening model in the Northern region of Portugal. Notwithstanding the high standards applied to the screening program, the elevated referral rates impose a significant burden to the health care system, which might compromise the cost-effectiveness of the program. Thus, alternative triage methods should be focused on investigation and implementation of continuous improvements.

Methylation markers as an alternative

Paper II described the potential alternative of methylation markers. Despite the heterogeneity of the studies analysed, methylation markers demonstrate high biomarker performance in different triage settings (e.g., clinician- versus self-collected samples), and, thus, constitute an alternative for triage of HrHPV-positive women in a population-based screening program.

Feasibility in the real-world screening samples

Paper III and **Paper IV** demonstrated the potential use of methylation markers in Northern Portugal's cervical cancer screening program. The methylation markers have shown to be an important alternative to LBC by reducing colposcopy referral and, most importantly, decreasing the number of women referred to colposcopic examination. Future studies should address the concerns regarding the management of HrHPV-positive women, which, according to the proposed triage algorithm, would not be referred to colposcopy. Defining the optimal management of these women is key to ensure safety and needs to be investigated as complement to the proposed strategy.

Nevertheless, the results obtained and discussed herein constitute, in our viewpoint, an important contribution to improve the Cervical Cancer Screening Program in Northern Portugal. The clinical validation of these results in a real-world context is the next logical step and of its results will depend on the diffusion to other regional screening programs in Portugal. Importantly, the implementation of methylation biomarkers to aid in the triage

of HrHPV-positive women also constitutes a unique opportunity to fully automatize the analytical pipeline of the screening program. This will enable the use of high-troughput and homogeneous technologies, increasing reliability in the results and improving cost-effectiveness. This new “ecosystem” will be in a privileged condition to welcome further innovation, such as the incorporation and progressive dissemination of self-sampling, increasing screening compliance and reaching the unscreened population, in which most cervical cancers will be diagnosed in the future.

Ultimately, the combined strengths of strategies for screening improvement are a pilar for the elimination of cervical cancer in Portugal, increasing compliance to screening programs and cost-effectiveness, reducing overdiagnosis and overtreatment, ensuring long term public acceptance and sustainability.