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Tese orientada pela Prof.ª Doutora Maria José G. G. Borrego, e co-orientada pelo Prof. Doutor Rogério Paulo de Andrade Tenreiro, especialmente elaborada para a obtenção do grau de doutor em Biologia (Especialidade em Microbiologia)

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INSTITUTO NACIONAL DE SAÚDE DOUTOR RICARDO JORGE

Departamento de Doenças Infecciosas

Laboratório Nacional de Referência para as Infecções Sexualmente Transmissíveis – Vírus do Papiloma Humano e Vírus Herpes genital

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'cause sometimes there's nothing else besides our FAITH,

This is for You!

You know I Love You... Forever!

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In accordance with Paragraph 1 of "Artigo 45, Capítulo V, do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, publicado no Diário da República – II Série No.65, de 30 de Março de 2012", it is clarified that full scientific articles already published (3), and under submission (1) for publication in peer-reviewed scientific journals, as well as for presentation purposes (4) were used in the elaboration of this dissertation. Hence, the candidate states that was involved in the study design, execution of experimental work, in the analysis and interpretation of results, and in their preparation for publication.

Resumo

A relação entre a infecção pelo Vírus do Papiloma Humano (HPV) e o desenvolvimento do cancro do colo do útero foi estabelecida no final do século XX. Em Portugal, preconiza-se o rastreio do cancro do colo do útero na mulher adulta por constituir uma das neoplasias mais frequentes. Foram recentemente implementados programas de rastreio numa base regional; contudo, o respectivo impacto na redução do número de casos de cancro do colo do útero levará algum tempo. Por outro lado, o recurso a potenciais indicadores de prognóstico, permitirá auxiliar a identificação precoce de mulheres em risco de desenvolver cancro do colo do útero, contribuindo para o estabelecimento de estratégias de prevenção mais efectivas e eficazes.

A vacina profiláctica contra a infecção por HPV foi recentemente disponibilizada para administração à população feminina jovem, tendo sido incluída no plano nacional de vacinação. Neste contexto, torna-se importante conhecer os genótipos circulantes na população Portuguesa, de forma a prever o impacto da vacinação (apenas inclui dois ou quatro genótipos de HPV) na infecção por HPV e nas lesões a esta associada.

O presente trabalho de doutoramento teve os seguintes objectivos: 1) determinação da proporção da infecção pelos diferentes genótipos de HPV numa amostra da população feminina Portuguesa (obtida por rastreio oportunista), e respectiva associação com o diagnóstico citológico; 2) avaliação dos testes de detecção e genotipagem do HPV relativamente à clínica associada; 3) avaliação de diferentes indicadores de prognóstico, de acordo com o diagnóstico clínico; e, 4) desenvolvimento de um modelo matemático aplicável ao estudo da infecção genital por HPV.

A determinação da frequência dos diferentes genótipos de HPV foi efectuada numa população com sinais clínicos sugestivos de infecção por HPV, tendo sido possível estabelecer associações significativas entre as alterações clínicas e a infecção persistente por genótipos de alto risco. Adicionalmente, foi possível identificar os genótipos mais frequentes, nomeadamente os HPV 16, 18, 31, 51, 53 e 66, e verificar que a infecção por HPV é mais frequente nas mulheres até aos 29 anos, sobretudo devido à multiplicidade de contactos e de parceiros sexuais (maioritariamente infecções transitórias). Já nas mulheres com idade superior a 30 anos, a infecção por HPV foi menos frequente mas apresentou maior risco de persistência e, dada a elevada proporção de genótipos de alto risco (mesmo em mulheres com citologia normal – assintomáticas), constituiu, por si só, um importante factor de risco para o desenvolvimento de cancro do colo do útero.

Foi efectuada uma caracterização epidemiológica da infecção por HPV, assim como dos programas de rastreio do cancro do colo do útero em Portugal, tendo sido salientadas as especificidades do rastreio em cada região.

O desempenho de diferentes testes comerciais de detecção e genotipagem do HPV foi avaliado de acordo com a clínica (sensibilidade, especificidade, valor preditivo positivo e negativo). A compreensão dos princípios e fundamentos de cada metodologia facilita uma correcta apreciação do respectivo desempenho laboratorial em fases distintas da infecção por HPV, assim como da sua aplicabilidade a programas de rastreio. Os testes de detecção do DNA viral, com elevada sensibilidade, revelaram constituir uma boa alternativa à recorrente citologia, enquanto teste de rastreio primário. De facto, a abordagem conjunta de teste HPV com triagem citológica, especialmente nas mulheres com mais de 30 anos, permitirá alargar o tempo de intervalo entre os exames de rastreio, devido ao elevado valor preditivo negativo do teste HPV. Mais, os testes de detecção do DNA viral, que incluem a genotipagem dos HPV 16 e 18 (oncogénicos e frequentemente associados ao desenvolvimento de lesões precursoras de cancro do colo do útero), possibilitam estratificar mais eficazmente as mulheres em maior risco de persistência da infecção viral e respectivas consequências clínicas.

Relativamente à detecção de RNAm do HPV, foi avaliada uma metodologia comercial de detecção e genotipagem de transcritos de RNAm para alguns genótipos de HPV de alto risco (NASBA). Esta metodologia permite a identificação precoce de infecções clinicamente relevantes por compreenderem um risco acrescido de desenvolvimento de lesões precursoras do cancro do colo do útero. Verificou-se que a utilização desta metodologia como teste de segunda linha pode aumentar a especificidade do teste de detecção de DNA do HPV nas mulheres infectadas, reduzindo a indicação clínica para colposcopia (que inflige elevada morbilidade e ansiedade na mulher) como método de rastreio das lesões associadas à infecção por HPV, e evitando o tratamento excessivo, ao possibilitar excluir lesões com maior probabilidade de regressão.

A utilização de diferentes indicadores de prognóstico da infecção por HPV facilitará a identificação precoce de mulheres em risco de desenvolvimento de lesões precursoras de cancro do colo do útero. Neste contexto, foram avaliadas a carga viral e o estado físico do DNA viral dos HPVs 16 e 18, tendo sido possível determinar uma associação entre o aumento da carga viral do HPV 16 e a gravidade da lesão do colo do útero, pelo que foi considerado como um importante marcador de prognóstico em mulheres infectadas por um dos mais frequentes genótipos de alto risco na população Portuguesa. Para o HPV 18, frequentemente associado ao desenvolvimento de adenocarcinomas (tipo de cancro cervical de difícil identificação citológica), verificou-se que a carga viral é potencialmente preditiva da persistência da infecção.

A determinação do estado físico do DNA viral, como metodologia alternativa a procedimentos médicos invasivos (colposcopia e biopsia), foi avaliada em associação com o diagnóstico citopatológico. Durante o processo de carcinogénese viral ocorre integração do genoma viral no genoma da célula hospedeira, por disrupção do gene viral E2 e subsequente sobre-expressão dos oncogenes virais E6 e E7. Foi possível identificar, especialmente para o HPV 18, uma associação entre a presença de formas lineares (maior risco), as lesões precursoras e os casos de adenocarcinoma, o que sugere a utilidade clínica deste indicador de prognóstico para as mulheres infectadas por HPV 18. No caso do HPV 16, a associação entre a determinação do estado físico do DNA viral e o diagnóstico citopatológico não foi tão evidente, pelo que outros mecanismos virais poderão estar associados à transformação maligna que antecede o desenvolvimento de cancro do colo do útero.

Por último, o desenvolvimento de um modelo matemático aplicado à infecção genital por HPV incluiu transições entre os diferentes estadios clínicos que correspondem ao processo de carcinogénese viral. Os cenários previstos foram extrapolados a partir da população de estudo (de rastreio oportunista) e posteriormente comparados com uma população Portuguesa de referência (de rastreio organizado), por forma a estimar a evolução e flutuações relacionadas com a infecção por genótipos de HPV de alto risco e respectivas lesões associadas. A utilização eficaz da vacina a nível mundial poderá levar a uma diminuição de casos de cancro do colo do útero na ordem dos 70% (valor estimado de cancros associados à infecção por HPV 16 e 18), decorrente da prevenção vacinal para os referidos HPVs. No entanto, a existência de uma proporção considerável de outros genótipos de alto risco não incluídos nas vacinas disponíveis poderá alterar esta estimativa, pelo que a monitorização constante dos genótipos circulantes de HPV será importante. De facto, estima-se que sejam necessárias algumas décadas até eliminar os casos de infecção associados aos HPV 16 e 18, já que a grande maioria das mulheres já foi exposta à infecção por HPV.

Palavras-chave: Portugal, Vírus do Papiloma Humano, cancro do colo do útero, rastreio, estratégias de prevenção.

Abstract

Cervical cancer development has been aetiologically linked to human papillomavirus (HPV) infection. In Portugal, routine screening for cervical cancer has been regionally implemented and was recommended for adult women because it constitutes one of the most frequent malignancies for women aged 15 to 44 years. HPV prophylactic vaccination (which only includes two or four oncogenic genotypes) was made available in the last decade for young girls, but the knowledge of HPV circulating genotypes is crucial for predicting its clinical impact.

This PhD thesis comprised the following objectives: 1) assessment of the proportion of HPV genotypes among Portuguese women (opportunistic screening) according to cytological diagnosis; 2) clinical evaluation of several HPV tests; 3) evaluation of some prognostic markers according to the clinical diagnosis; 4) development of a mathematical model applied to genital HPV infection.

Portuguese HPV epidemiology and screening programs were characterized, highlighting the specificities of screening in each region. The most frequent genotypes were HPV 16, 18, 31, 51, 53 and 66, and HPV infection was more common in women aged less than 29 years. In women over 30 years, HPV infection was less frequent but tend to persist and to involve high-risk genotypes.

HPV DNA tests demonstrated high sensitivity, constituting an alternative to cytology in primary screening, while combined with cytology (especially for women over 30 years) would extend the re-screening testing interval, considering the high negative predictive value of HPV testing. Moreover, HPV DNA tests with concurrent identification of HPV 16 and 18 (most associated genotypes to cervical cancer development) will provide a better risk stratification of women for precancerous cervical lesion development.

The detection of HPV mRNA is highly specific in identifying clinical cervical disease, so that its recommendation to reflex testing of HPV DNA-positive women has shown to reduce colposcopy referral, avoiding over-treatment by excluding cervical lesions that would most likely regress (with associated morbidity and anxiety to HPV-infected women).

The viral load and physical status of high-risk HPV 16 and 18 were evaluated as prognostic markers in women at risk of developing cervical precancerous lesions. An association between increased HPV 16 viral load and severity of cervical lesion was observed suggesting its prognostic value, whereas for HPV 18, viral load was only predictive of HPV persistency. The presence of linear forms (higher risk) in HPV 18-associated precancerous lesions and adenocarcinomas evidenced the potential clinical utility of viral DNA physical status as a prognostic marker for women infected with HPV 18. The association was not evident for HPV 16, suggesting that other viral mechanisms should be responsible for malignant host cell transformation.

Finally, the mathematical model applied to genital HPV infection included transition probabilities between disease states corresponding to different steps of cervical carcinogenesis, and provided scenarios for a Study Population (opportunistic screening), for a Reference Population (routine screening) and for a Hypothetical Population, which were further compared to predict HPV vaccination impact. Estimates of trends and fluctuations associated with high-risk HPV genotype infections and its associated cervical lesions were performed. HPV vaccines may lead to a global decrease of cervical cancer cases of about 70%, considering that most cervical cancers are associated with HPV 16 and 18 infections. However, the existence of a considerable proportion of other high-risk genotypes not included in the currently available HPV vaccines may change this estimate. Thus, the constant monitoring of circulating HPV genotypes remains of particular importance. Furthermore, the vast majority of women have been exposed to HPV infection, alerting to the importance of a continuous follow-up and establishment of public health measures through routine screening, while improving women's welfare.

Keywords: Portugal, human papillomavirus, cervical cancer, screening, prevention strategies.

List of Publications

This PhD thesis comprehends the following papers, which will be referred to in the text by their Roman numerals:

I. Gradíssimo A, Verdasca N. Cervical Cancer Screening in Portugal: A Perspective (in submission) (2013).

II. Pista Â, Verdasca N, Oliveira A. Clinical performance of the CLART Human Papillomavirus 2 assay compared with the Hybrid Capture 2. J Med Virol 2011;83(2):272-276.

III. Oliveira A, Verdasca N, Pista Â. Use of NucliSENS EasyQ HPV assay in the management of cervical intraepithelial neoplasia. J Med Virol 2013;85(7):1235-1241.

IV. Gradíssimo Oliveira A*, Delgado C*, Verdasca N, Pista Â. Prognostic value of human papillomavirus types 16 and 18 DNA physical status in cervical intraepithelial neoplasia. Clin Microbiol Inf 2013 May 9. DOI: 10.1111/1469-0691.12233 [Epub ahead of print]. (*contributed equally).

Notes of the Author

This PhD thesis comprehends the detailed description of an extended work mainly focused on how to improve the existent screening strategies applied to the Portuguese female population, on how to better manage HPV-infected women, while reducing the associated anxiety and morbidity.

The presentation order does not follow a chronological sequence according to publication but it is rather intended to present a comprehensive approach in view of the objectives. First is presented data that correspond to baseline information to the following presented work, from the general to the particular point of view.

Since all published papers are presented here in a PDF format (original layout), following the guidelines from each international journal where they were published, references are not uniformized throughout the PhD thesis. Regarding non-published chapters, references follow the guidelines from the international journal Clinical Microbiology and Infection (Vancouver style).

All chapters are presented in an article structure, so abbreviations may be defined more than once, and every chapter includes a specific reference section. When Paper IV was submitted the full-case analysis was not completed, therefore updated results are now presented in Chapter 8.

The author chose not to follow the new spelling agreement for Portuguese language in the *Resumo* section. The author follows USA English spelling throughout this PhD thesis. The fellowship institution, *Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.*, will be further addressed in the text as National Institute of Health (INSA).

CONTENTS

Abbreviations

In this thesis acronyms are expanded on first usage and whenever deemed necessary to improve clarity.

6-FAM	6-carboxy fluorescein
6-ROX	6-carboxy X-rhodamine
AIN	Anal Intraepithelial Neoplasia
ARS	Administração Regional de Saúde (Regional Health Administration)
ASC-US/ASCUS	Atypical Squamous Cells of Undetermined Significance
ASC-H	Atypical Squamous Cells "cannot exclude HSIL"
BPV	Bovine Papillomavirus
CFTR	*cystic fibrosis transmembrane conductance regulator*
CI	Confidence Interval
CIN	Cervical Intraepithelial Neoplasia
CIN2+	Cervical Intraepithelial Neoplasia grade 2 or worse
CIN3+	Cervical Intraepithelial Neoplasia grade 3 or worse
CLART	CLART® Papillomavirus Humano 2
CLEAR	Clinical Evaluation of APTIMA HPV RNA
CLEOPATRE	the Cervical Lesions Observed by Papillomavirus Types – a Research in Europe
DGS	Direcção Geral de Saúde (General Directorate of Health)
DTS	Direct-Tube Sampling
E	Early region
FCT	Fundação para a Ciência e Tecnologia
FDA	Food and Drug Administration
HBA	Hospital do Barlavento Algarvio (Barlavento Algarvio Hospital)
HC2	Hybrid Capture® 2 HR HPV DNA Test
HF	Hospital de Faro (Faro Hospital)
HIM	Human papillomavirus Infection in Men
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
HR	High-risk
HR-HR	multiple infection with exclusively high-risk HPV genotypes
HR-LR	multiple infection with high-risk and low-risk HPV genotypes
HR-HPV	High-Risk Human Papillomavirus
HSIL	High-grade Squamous Intraepithelial Lesion
HSV	Herpes Simplex Virus
HUC	Hospitais da Universidade de Coimbra Coimbra University Hospitals
IARC	International Agency for Research on Cancer
ICC	Invasive Cervical Cancer
ICTV	International Committee on the Taxonomy of Viruses
INSA	Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P. (National Institute of Health)
IPO Coimbra	Instituto Português de Oncologia de Coimbra (Coimbra Portuguese Oncology Institute)
IPO Lisboa	Instituto Português de Oncologia de Lisboa (Lisbon Portuguese Oncology Institute)
IPO Porto	Instituto Português de Oncologia do Porto (Oporto Portuguese Oncology Institute)
L	Late region
LBC	Liquid-Based Cytology
LCR	Long Control Region
LR	Low-Risk
LR-LR	multiple infection with exclusively low-risk HPV genotypes
LR-HPV	Low-Risk Human Papillomavirus
LSIL	Low-grade Squamous Intraepithelial Lesion
M	Multiple infections

MSM	Men having Sex with Men
MSW	Men having Sex with Women
NILM	Normal epithelium
NPV	Negative Predictive Value
OR	Odds Ratio
ORF	Open Reading Frame
Pap	Papanicolau smear
PIN	Penile Intraepithelial Neoplasia
PPV	Positive Predictive Value
pRB	Retinoblastoma protein
RLU	Relative Light Units
RRP	Recurrent Respiratory Papillomatosis
S	Single infections
SCC	Squamous Cell Carcinoma
SD	Standard Deviation
SPSS	Statistical Package for the Social Sciences
STI	Sexual Transmitted Infection
TCR	T Cell Receptor
U1A	U1 small nuclear ribonucleoprotein
VaIN	Vaginal Intraepithelial Neoplasia
VIN	Vulvar Intraepithelial Neoplasia
VLP	Virus-Like Particles
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Aetiological Role of Human Papillomavirus in Cervical Cancer

zur Hausen [1,2] turned all eyes and interest into human papillomavirus (HPV) research after repeated and unsuccessful attempts to provide a decisive evidence for an aetiological role of herpes simplex virus type 2 (HSV 2) in cervical cancer carcinogenesis, and he formulated the hypothesis that HPV would be related to cervical cancer. During the 80's, the identification of some genotypes of papillomavirus in the anogenital tract [3] led to an intensive research in the field, which culminated in the development of clinical tests for cervical cancer based on HPV DNA detection, and in the conception of vaccines that preclude HPV infections in the present.

Years of research provided the conclusion that virtually all cervical cancers are related to HPV infection [4,5], so that cervical cancer is the first cancer that is acknowledged by the World Health Organization (WHO) to be virally induced in essentially all cancer cases.

Cervical cancer is, after breast cancer, the second most common malignancy among women worldwide. The estimated total number of women diagnosed with cervical cancer in 2002 was 493,000 and 274,000 women died from the disease that same year. The majority of cases (83.0%) occur in developing countries, where cervical cancer accounts for 15.0% of the newly diagnosed cancers in women. In developed countries comprehending good screening options, invasive cervical carcinoma (ICC) is a relatively rare condition and accounts for only 3.6% of the new cancers [6]. The 2008 worldwide estimates[1] revealed that the incidence of cervical cancer was 15.2 per 100,000 women (530,232 new cases) and the mortality rate was 7.8 per 100,000 (275,008 deaths) [7].

In Portugal, cervical cancer is the fourth most frequent cancer in women of all ages, and the second most frequent in women between 15 and 44 years of age. In 2008, 949 new cases of cervical cancer were diagnosed, with an age-standardized incidence rate of 12.2 new cases per 100,000 women, and a total of 346 women died as a consequence of cervical cancer, with an age-standardized mortality rate of 3.6 women per 100,000 [7].

[1] GLOBOCAN is a project that estimates worldwide Incidence, Mortality, Prevalence and Disability-adjusted Life Years (DALY's) from major type cancers at national level; it is developed by the International Agency for Research on Cancer (IARC).

Classification and Structural Organization

Papillomaviruses are non-enveloped double-stranded circular deoxyribonucleic acid (dsDNA) viruses that comprise about 8000 base pairs (bp) in length. The capsid presents an icosahedral structure composed by 72 capsomers, and a diameter of 52-55 nm [8]. The genomes of all HPV genotypes contain approximately eight open reading frames (ORF), divided into three functional parts: the early (E) region that encodes proteins (E1-E7) necessary for viral replication; the late (L) region that encodes the structural proteins of the capsid (L1-L2) required for virion assembly of new particles; and a non-coding region that is referred to as long control region (LCR), which contains *cis* elements that are necessary for the replication and transcription of viral DNA (Fig.1) [9,10]. Early proteins E5, E6, and E7 are involved in promoting host cell proliferation and survival, and E6 and E7 play a key role in HPV-associated carcinogenesis. Three other early proteins (E1, E2, and E4) are involved in the control of viral gene transcription and viral DNA replication [5,8].

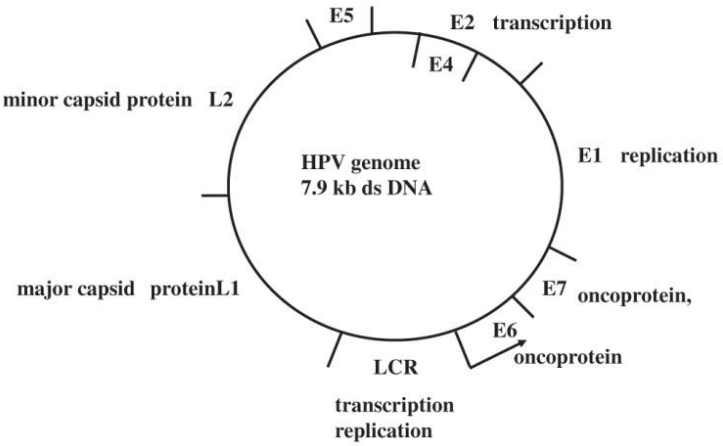


Fig. 1: Human papillomavirus (HPV) genome organization [10].

Papillomaviruses had originally been grouped together with polyomaviruses in one family, the *Papovaviridae*. It was later recognized that the two groups of viruses shared less features than expected, being now officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families – *Papillomaviridae* and *Polyomaviridae* [11]. Papillomaviruses are designated using the name of their host species, like bovine papillomavirus (BPV) or human papillomavirus (HPV). Further taxonomic organization encompasses attributing a number to each papillomavirus identified in the same host, e.g. HPV 1 and HPV 2, being designated as papillomavirus genotypes [10]. The identification of a novel genotype used to be determined through restriction enzyme electrophoresis profile. More recently, only the complete sequencing of the full genome may determine a new papillomavirus genotype, mainly based on the comparison of

the L1 gene sequences, while grouping them in genera, species and types (Fig.2). Since the L1 ORF is the most conserved region within the genome, a new papillomavirus isolate is recognized if the DNA sequence of the L1 ORF differs by more than 10.0% from the closest known genotype. Differences in homology between 2.0% and 10.0% define a subtype and those of less than 2.0% define a variant [11]. The taxonomic classification also relates to both tropism and viral properties. The phylogenetic trees for papillomaviruses evidence an evolutionary diversification in which an unknown molecular mechanism seems to restrict papillomaviruses to their host species, since it has been proposed that viral replication is linked to epithelial host cell replication, resulting in similar rates of nucleotide exchanges [10].

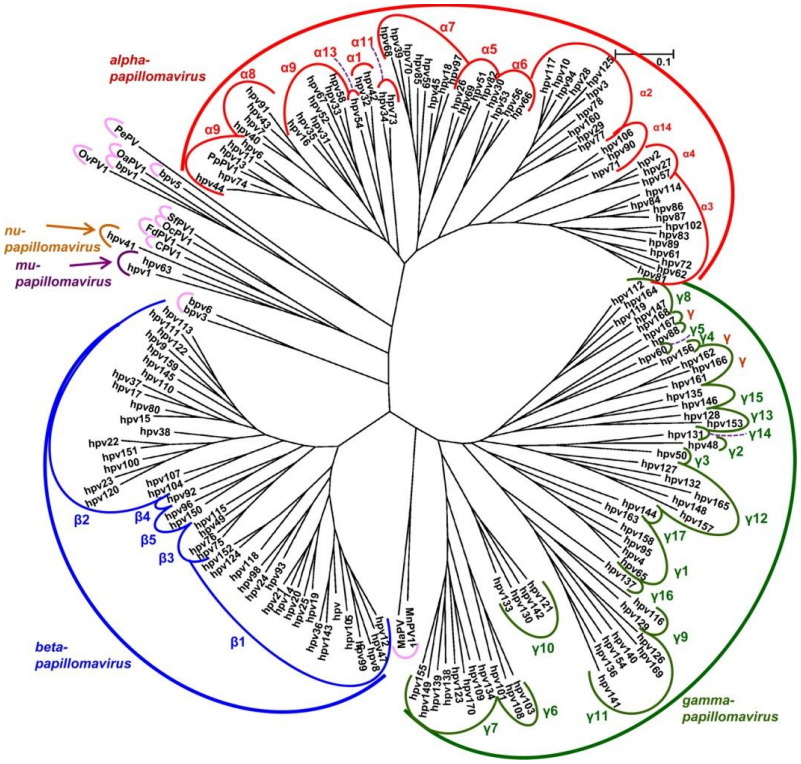


Fig. 2: Phylogenetic tree inferred from the L1 nucleotide sequences of 170 papillomaviruses [12].

More than 120 HPV genotypes have been identified so far and approximately 40 different genotypes can infect the anogenital tract. According to the oncogenic potential, HPV can be classified into low-risk genotypes (LR-HPV), mainly seen in genital warts and non-progressing low-grade lesions [13,14]. According to the International Agency for Research on Cancer (IARC) Monograph Working Group, at least 13 HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66) are considered as high-risk (HR-HPV) [15,16].

Viral Life-Cycle and Oncogenesis

Throughout the years, HPVs have co-evolved with their host, with most of them being able to be maintained in the population without causing any apparent disease [17,18]. This co-evolution raises the hypothesis of a balance between viral replication and immune tolerance [19]. In fact, the different epithelial diseases caused by HPV genotypes appear to be linked to their strategies of transmission and propagation within the epithelium, as well as different interactions with the immune system. HPVs can be divided into cutaneous and mucosal genotypes, presenting a specific tropism to infect epithelial cells and have adapted their life-cycle in accordance to host cell differentiation [20,21].

The low-risk HPV genotypes present a life-cycle organization which is not able to cause neoplasia [22]. The main differences are found in the promoter positioning and its regulation, as well as in messenger ribonucleic acid (mRNA) splicing, mainly affecting E6 and E7 protein expression [20,23]. Since these proteins are involved in the carcinogenesis process, in low-risk genotypes, such as HPV 6, the E6 gene seems to not play a key role in inducing host genome instability, affecting the disease phenotype following infection [24,25]. For high-risk HPV genotypes, they seem to be preserved through their ability to persist and cause productive infections, promoting cell proliferation in the basal cell layer of the epithelium (primary site of infection and reservoir during persistence) [26,27]. High-grade lesions correspond to abortive infections, whereas an elevated expression of the E6 and E7 proteins is observed which is closely related to an increasing severity of neoplasia [22,28,29].

High-risk HPV infection is thought to require the presence of a microwound that allows the infectious virions to access the basal cell layer of the epithelium, where the viral genome will be maintained in a low copy number [30,31]. However, multiple entry pathways have been proposed, depending on the genotype of virus or the route of infection. Cell entry has been proposed to occur through an interaction implicating heparin sulphate proteoglycans [32-34] and Alpha 6 Integrin [35-40].

HPV infections do not present viraemic phase, and genome amplification often occurs in the initial stages of infection followed by maintenance of the viral episome at a low copy number [30,41,42]. In this initial amplification phase, the viral proteins E1 and E2 are crucial in regulating viral transcription through various binding sites in LCR [23,43-46]. The oncogenic potential of a papillomavirus is linked to its ability to overcome growth arrest and maintain a replication-competent cell [47]. Expression of the viral E6 and E7 proteins during the next phase of infection delays host cell cycle arrests and differentiation (normally observed as epithelial cells move up from the basement membrane to become mature keratinocytes). The E7 protein, which has the ability to

associate to proteins of the retinoblastoma (pRB) family, enables the virus to manage the control of the cell cycle in the upper epithelial layer [27,48]. Of the interactions attributed to high-risk E6 protein, the capacity to upregulate telomerase activity [49-51] and to maintain telomerase integrity during repeated cell divisions, together with their ability to mediate the degradation of p53 within the cell, seems to be the most important. This expression of E6 and E7 proteins in the upper epithelial layer allows the infected cell to re-enter S-phase, elevating the viral copy number, and usually thickening the skin, which is characteristic of some papillomavirus infections. [20,52]. In addition to E1 and E2 expression, E4 and E5 proteins are also involved in the viral genome amplification through the modification of the cellular environment, where E5 protein is particularly involved in koilocyte formation [53].

When the differentiation of replicating epithelial cells to non-replicating mature keratinocytes eventually occurs (in the most superficial layer of epithelial cells), the final step of the viral life-cycle mainly involves the expression of the minor coat protein L2, leading to the exit from the host cell, and the expression of the major coat protein L1 that culminates in the final genome packaging [54,55]. This event contributes to the production of very stable infectious virions [56,57]. In this stage, E4 protein enables virion release and infectivity in the upper epithelial layer [21]. Viral genome encapsidation requires the assembly of the icosahedral capsid in the host nucleous, prior to L1 expression [58,59]. Viral particles are then released from the host cell in the upper layers of the epithelium through a nonlytic process (Fig.3). New virus particles may infect new cells of the same host or be transmitted to new hosts through sexual contact [20].

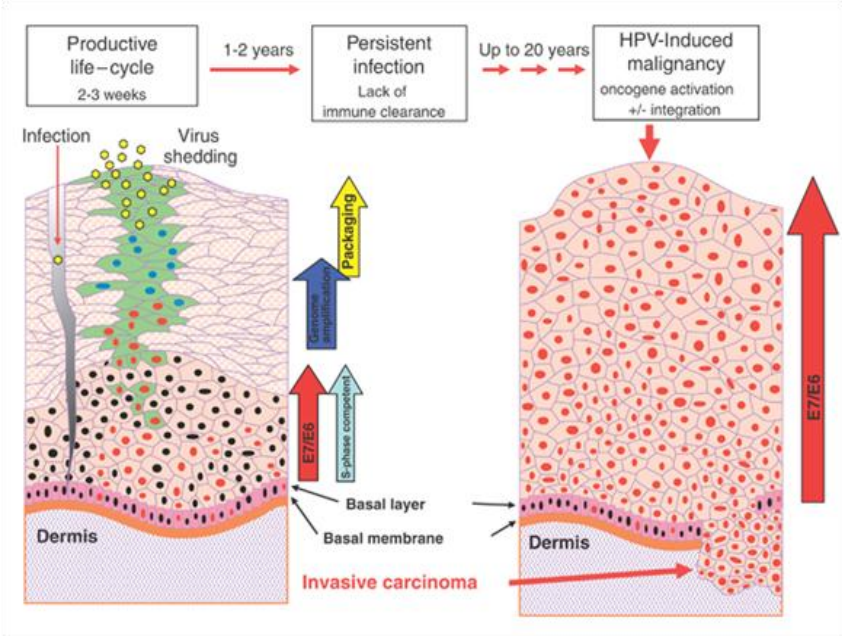


Fig. 3: HPV infectious cycle scheme: Virus entry in a cell through a microabrasion and infection beginning with binding of HPV particles to the basal cells of the epithelium, where the viral genome will be amplified to several copies. In the suprabasal layers of the epithelium, HPV early proteins are expressed and viral replication will take place. E4 and the late L1 and L2 proteins will be expressed only in the most superficial layers of the epithelium, where HPV DNA will be encapsidated, and the virions released from the epithelial surface [60].

In women with cervical HPV infection the virus particle production becomes disrupted by the increase in the expression levels of E6 and E7 from cervical intraepithelial neoplasia grade 1 to 3 (CIN1 to CIN3), which directly alters the neoplastic phenotype. The overexpression of E6 and E7 oncoproteins detected in HR-HPV infections that lead to CIN 2 or worse (CIN2+) development, which is linked to the accumulation of genetic changes in the host cell, are important to cancer progression [61-63]. However, most of the HPV infections are cleared and do not persist long enough to occur viral deregulation, and consequently induce genetic errors in the infected host cell. HPV 16 is associated with a longer persistence of infection than other HR genotypes which can contribute to its higher cancer risk [64,65].

The HPV transmission rate reported as the transmission probability per partnership (v.g. the probability that an infected partner transmits HPV to a susceptible partner irrespective of the duration of that partnership) has been evaluated through longitudinal studies [66,67]. It has been consistently reported a higher rate of female-to-male versus male-to-female transmission (0.19-0.81 vs 0.05-0.28, respectively), being higher in short-term interval study visits [68]. Bogaards *et al*. [69] estimated transmission probabilities ranging 0.43-0.94, whereas the highest were for HPV 16 and 18. One study revealed that the highest transmission rate occurs when one of the partners have persistent HPV infection, suggesting that transmission is linked with extended exposure and high viral load [67]. However, due to recent sexual intercourse (prior to study visit), some false HPV positivity may over-estimate transmission rates [70].

Natural Immune Response

The exclusively intraepithelial nature of the HPV infection without viraemia or virus-induced cell death stages is not associated with inflammation and thus, may suggest that presentation of antigen to lymph nodes is limited [71,72], resulting in a failure to induce an effective immune response, due to inefficient activation of innate immunity and ineffective priming of the adaptive immune response. This defective immune response may facilitate viral persistence, a key feature for evasion of HR-HPV infections [73]. However, a vast majority of the cervical HPV infections are transient, clearing within 1 to 2 years [74]. This suggests that the host immune system somehow plays a role in preventing or eliminating HPV infection, through both humoral and cell-mediated responses [75]. It has been recently proposed that protection against infection (preventing the virus entry in the basal epithelial host cells) would be accomplished through neutralizing antibodies, while clearance is thought to be mediated predominantly by cellular immunity [76]. When one HPV infection is successively detected in the same woman it is hard to determine if it is due to re-infection

after an effective clearance, or if it is due to a reactivation of the same latent infection. Moscicki *et al*. [27th International Papillomavirus Conference and Clinical Workshops, abstract O-08.06] showed that after two years, only 3.0 to 5.0% of the women evidenced re-infection with HPV 16, following clearance of the first infection (defined as two consecutive negative HPV DNA results), and that after five years past, the re-infection rate increased to the range of 10.0 to 17.0%. The results of this study are supported by the findings of Trottier *et al*. [77], which also evidenced differential sexual behaviors highly associated with repeated detection of the same HPV genotype infections, suggesting that each new infection is probably due to re-exposure. There are some evidences supporting that transient infections could be cleared by innate immune responses [78], which do not provide memory immune response, leaving women vulnerable to re-infection, but rarely leading to cervical lesion development [79]. Re-infection rates should not be based on serologic evidence, because serology lacks HPV-genotype specificity, leading to putative contradictory data on whether antibodies are protective regarding re-infection or not [27th International Papillomavirus Conference and Clinical Workshops, abstract O-08.06;77,80,81].

To date, most of the seroepidemiological data on HPV infection have been obtained from the populational groups included in the ongoing HPV vaccine trials, in addition to some natural history studies held in some cohorts [82-84]. Despite the number of seroepidemiological reports, the detailed kinetics of the various components of the humoral immune response following incident HPV infection in naïve women remains unclear and somewhat controversial. In fact, although serum IgG antibodies to HPV 16 are found in 56.0 to 60.0% of subjects with incident HPV infections within 8 to 18 months, antibody titers remain low [83,85], but can persist for decades, or rapidly disappear in women without HPV-associated lesions [85]. In contrast to the naturally occurring humoral response, vaccination with HPV L1 virus-like particles (VLP) has shown to induce high titers of virus-specific serum IgG antibodies and protection against virus-induced (pre)malignant lesions [86,87].

Immune cells infiltrate the HPV-associated lesion but, during persistent infection, they fail to access the infected epithelium. On the other hand, when CD8+ T-cells reach the neoplastic lesion is more likely to regress [88]. Circulating T-cells with the ability to access the infected epithelium are mediated by chemokines and adhesion molecules [89]. Finally, high-grade lesions and cancer cells can lose the expression of human leukocyte antigen (HLA) molecules and immune cells will be inefficient as target cells will no longer exhibit cell surface HLA-HPV peptide complexes recognizable to the T-cell receptor (TCR). Considering this, therapeutic vaccines that would induce or boost HPV T-cell adaptive immunity could be of value [73]. However, therapeutic vaccines have been tested in HPV 16-associated diseases without significant clinical impact, probably because they fail to induce the necessary effective HPV-specific CD4+ and CD8+ T cell responses [90].

Epidemiology of HPV-related Diseases

Benign Diseases

Recurrent respiratory papillomatosis (RRP) and genital warts, which are unequivocally linked to HPV 6 and 11 infections that are responsible for 96.0 to 100% of all genital warts cases, constitute highly recurrent benign HPV-associated diseases [91-94].

In developed countries, genital warts present epidemiological trends similar to other common sexually transmitted infections (STIs) with a peak of incidence among individuals younger than 25 years [95]. An eight-fold increase in the incidence of genital warts was observed in the last decades [96-98], probably associated with changes in sexual behavior, namely the lower age at sexual debut reported by many developed countries [99]. Several recent publications indicate an annual incidence of 0.1 to 0.2% with a peak occurring among teenagers and young adults [100-103]. Also, the incidence of genital warts seems to be higher in human immunodeficiency virus (HIV)-positive women [104]. There is now accumulating evidence that population-based vaccination can result in a high decrease in genital warts incidence, with reduction of HPV 6/11 burden [103]. Nonetheless, the available treatment options often face recurrence of these benign lesions and imply significant public health and emotional costs [93].

RRP is caused primarily by HPV 6 and 11, with a small fraction (less than 5.0%) caused by HPV 16 or other genotypes [105]. The disease is characterized by the growth of multiple papillomas, usually arising from the larynx, and affects mainly in early childhood (juvenile onset). Nonetheless, RRP is a rare disorder, with a prevalence of 3 to 7 per 100,000 for both pediatric and adults [106,107]. The most important risk factor for the juvenile onset RRP is a maternal history of genital warts during pregnancy, for whom it has been estimated 200 times greater risk of associated RRP in the infant when compared to women without genital warts upon natural birth [108]. Adult onset RRP has been associated with high lifetime number of sexual partners and oral sex [109], as established for oropharyngeal cancers. Recurrence rates are highly variable, being more associated with young age [110], and the vast majority is benign. However, malignant conversion may occur when pulmonary involvement is observed and usually caused by HPV 11 infection [111].

Head and Neck Cancers

Head and neck cancers are commonly referred to squamous cell carcinomas (SCCs) that are diagnosed in the upper digestive and respiratory tracts, affecting the oral cavity, tonsils, base of the tongue, oropharynx, and larynx. In 2002, approximately 405,000 new cases were reported causing

221,000 deaths worldwide [112], mainly among older men and women (over 60 years) [113]. Tobacco use and alcohol consumption constitute potential risk factors for the development of head and neck cancers [103]. However, 15.0% to 20.0% of the cases of head and neck cancer have no association with these risk factors [113].

In SCC, the prevalence of HPV DNA depends on the type of study and geography, but an overall prevalence of HPV DNA of 25.9% (23.5% for oral cavity tumors; 24.0% for larynx tumors; and 35.6% for oropharynx tumors) has been proposed [114]. The detection of oral HPV infection varies among studies, depending greatly on sampling technique. A meta-analysis of 4581 cancer-free individuals from 18 different studies established an HPV prevalence of 4.5% [115], but for a population-based survey held in the United States of America (USA), the HPV prevalence was estimated in 6.9%, (higher HPV positivity in men than in women), and was associated with changes in sexual behavior (v.g. oral sex) [93,116,117]. HPV 16 is the most commonly detected HPV genotype in head and neck lesions followed by HPV 18 [114,116,118]. The IARC Monograph [15] established the average prevalence of HPV infection in the oral cavity in approximately 25.0%, with HPV 16 being detected in about 70.0% of the HPV-positive cases. The average prevalence was higher for oropharyngeal cancers (35.0%) with HPV 16 being detected in 80.0% of the HPV-positive cases, suggesting an aetiological association of this genotype with this type of cancer [110]. Sexually transmitted oral HPV infection was proposed as the major cause of the HPV-associated head and neck cancers; however, transmission can also occur through open-mouth kissing or hand to mouth inoculation [117,119]. Sexual behaviors most consistently associated with head and neck cancers risk include high lifetime number of sexual partners (independently of oral or genital intercourse). Less frequently, it has been also suggested the influence of younger age at sexual debut, lack of condom use, same-gender sexual contacts and oral-genital or oral-anal sexual practices [110].

Penile Cancer

In men, HPV DNA is frequently detected among penile cancer cases (40.0-45.0%) [93,120]. HPV is more frequently detected in the shaft, glans, and scrotum, whereas less in the urethra. Male genital HPV prevalence is usually related to female prevalence in the same population [121]. The HPV positivity rates in men vary less by age group than the observed for women. However, the detection of genital HPV infection in men is influenced by cell sampling techniques [122]. In the Human Papillomavirus Infection in Men (HIM) study, in which samples from the coronal sulcus/glans, penile shaft and scrotum were combined, the prevalence of any HPV genotype was 65.2%, whereas 20.7% were LR-HPV genotypes and only 17.8% were HR-HPV genotypes, and no association with age could be established [123]. In other study, HPV prevalence was higher among men having sex with men (MSM) than in men having sex with women (MSW) (23.0% vs. 8.0%,

respectively) [124]. The prevalence of HPV in penile intraepithelial neoplasia (PIN) is higher than the reported for invasive cancer of the penis [122]. Several cross-sectional studies have provided evidence of risk factors for the acquisition of HPV infection in male genitalia including "not being circumcised" [125], "lack of condom use" [126], "history of tobacco smoking habits" [127], and a "high number of lifetime partners" [123,128].

Anal Cancer

Anal cancers are rare malignancies that affect the transitional zone of the epithelium in the anal canal. Worldwide, approximately 99,000 new cases of anal cancer were reported in 2002, among which 40.0% in men and 60.0% in women. An increasing trend in the incidence rate of this cancer (about 2.0% per year) has been reported, affecting both men and women, but affecting particularly MSM and HIV-infected men and women [112,129-131]. Several studies reported that over 90.0% of the HIV-infected MSM have anal HPV infection, often with multiple genotypes (at least 7.0% evidenced one HR-HPV genotype), while a substantially lower proportion of HIV-uninfected MSW have anal HPV infection (12.0%) [132]. Studies conducted in women with and without HIV infection reported that anal HPV infection is more common than cervical HPV infection [133,134]. The majority of invasive anal carcinomas (65.0%) are SCC that develops from precancerous anal intraepithelial neoplasia (AIN) lesions. For both sexes, around 88.0 to 94.0% of the cases of anal cancer are associated with HPV infection, but the highest prevalence is observed in MSM (73.5%) [103,131,135]. The most common HPV genotype detected amongst anal cancer cases is HPV 16 (up to 87.0%), followed by HPV 18 (9.0%) [15]. HPV DNA is also detected in AIN lesions and the prevalence of the HPV infection increases with the severity of the lesion (75.0% in AIN1, 86.0% in AIN2, and 94.0% in AIN3) [136]. The increasing incidence rate of anal cancer may reflect changes in sexual behavior during the second half of the twentieth century that might have increased the risk for exposure of the anal canal to the HPV infection, considering that anal intercourse became a more frequent sexual practice and this is a more efficient mode of HPV acquisition [137]. However, it has been considered that anal intercourse is not essential for anal HPV infection, as it might spread from cervical HPV infection [133,138].

Vulvar and Vaginal Cancer

HPV infection has been associated to various cancerous lesions in the female anogenital tract. HPV DNA has been detected in vaginal cancer (64.0-91.0%), and its precursor vaginal intraepithelial neoplasia grade 3 (VaIN3) lesions (82.0-100%). Similarly, an estimated 40.0 to 50.0% of the vulvar

cancer has also been associated to HPV [93,139]. In worldwide estimates from 2008, the age-standardized incidence rate of vulvar and vaginal cancer did not exceed 2 per 100,000, despite the limitations for estimating a trend in the incidence of these types of cancer due to underreporting in developing regions [103]. Moreover, the incidence of invasive vulvar and vaginal cancer was reported as stable over time signalling the limited impact of cervical cancer screening efforts on other genital cancers [93]. This might be linked to the fact that vulvar and vaginal cancers usually affect women older than 65 years, age at which women normally exit a cervical cancer screening program.

Vulvar cancer is thought to be a rather uncommon malignancy, affecting 26,800 women worldwide in 2002, which accounted for approximately only 3.0% of all gynaecological cancers [140]. SCCs constitute the most common (90.0%) histologic type of primary vulvar cancer, and about 60.0% of the cases were reported in developed countries [140,141], where they were more common among older women, with approximately 66.0% of the cases being diagnosed in women aged 70 years or older. Regarding genotype-specific HPV prevalence in vulvar lesions, 67.0% relate to LR-HPV-positivity in vulvar intraepithelial neoplasia grade 1 (VIN1) lesions, whereas only 6.0% of the HPV DNA-positive specimens were HPV 16. In contrast, 100% of VIN3 lesions were HR-HPV-positive and the vast majority (91.0%) were HPV 16-positive [142]. According to IARC Monograph [15], HPV DNA-positivity ranges from 72.0 to 100% for VIN3 lesions and from 27.3 to 100% in vulvar cancer, with HPV 16 being the most commonly detected genotype (65.0-93.0% in VIN and 72.0% for vulvar cancer) followed by HPV 18.

Cancer of the vagina has been a rare condition. In 2002, approximately 13,200 women worldwide were diagnosed with vaginal cancer, accounting for less than 2.0% of all gynaecological cancers [140]. SCCs are the most common histological type (~90.0%), with the vast majority (68.0%) being reported in developed countries [141]. Incidence rates in the range of 0.4 to 0.7 per 100,000 were reported in the USA, the United Kingdom (UK) and other European countries [143]. An increasing trend in the incidence of VaIN has been observed for the last decades, while the incidence rate of invasive vaginal cancer was reported to remain stable [144] or even evidencing some decline [145]. This type of cancer is usually diagnosed in older women (median age of 69 years; incidence peak at 55-70 years), being rare in women under 45 years [146-148].

Cervical Cancer[2]

The aetiological role of HPV in cervical cancer has been firmly established biologically and epidemiologically, as 99.7% of all cervical cancers actually containing oncogenic forms of the virus

[2] The Portuguese prevalence estimates will be described in Chapter 2, due to the importance of epidemiologic data for the objectives aimed in this PhD thesis.

[4,149,150]. Two HR-HPV genotypes, HPV 16 and 18 have been consistently associated to at least 70.0% of the cervical cancer cases worldwide, with limited geographical variability [5,151].

Estimates from GLOBOCAN 2008 [152,153] evidenced that of the 12.7 million of new cancers occurring worldwide, 700,000 occurred at an HPV-associated cancer anatomic site (cervix uteri, anus, penis, vulva, vagina, and oropharynx) and it was estimated that 610,000 of those were attributable to HPV. This represents 4.8% of the total cancer burden worldwide, which is lower than the 5.2% previously estimated in 2002 [112]. However, there was an increase of the estimated new cases from 561,000 to 610,000. As expected, 490,000 cases attributable to HPV (80.6% of the total) occurred in less developed regions (6.9%), whereas 2.1% were described in more developed regions [154], which could be predicted by the differential access to cervical cancer screening.

Cervical cancer represents the final step of a persistent unresolved HPV infection. The later are highly common, especially in young women among who the probability of ever be infected by HPV is around 80.0 to 90.0%, considering the 40.0 to 80.0% prevalence observed in many developed countries. As the majority of these infections will clear spontaneously without the manifestation of any clinical signs, it is estimated that only a fraction of 4.0 to 10.0% will be considered persistent HPV infections [137,155]. In cohorts of female university students in the USA and Canada, followed through repeated cervical sampling, the cumulative incidence of HPV infection was established in 40.0 to 60.0% among women that were negative at baseline during three years of follow-up [156-158]. In a cohort of initially HPV-negative women aged 15 to 19 years in England, the cumulative risk for HPV infection was 44.0% after three years and 60.0% after five years [159].

The overall global burden of HPV infection has been assessed by using pooled data from studies of HPV detection in women with normal cytology. The most recent meta-analysis, including data from 194 studies worldwide, estimates the global HPV prevalence in 11.0 to 12.0% [160]. There is a considerable regional variation, comprehending higher HPV prevalence rates in less developed countries. Regarding age, the HPV prevalence is globally higher in young women, increasing each year from 14 to 24 years and then declining rapidly with increasing age [161-164]. The highest prevalence is observed in women aged 20 to 24 years (44.8%) (24.5% for women aged 14-19 years and 27.4% among women aged 25-29 years) [161,162,165-168]. However, in Asian and African populations, HPV prevalence although decreasing after 25 years, increases again among middle-aged women [164]. If we look further into HPV genotype-specific data in the meta-analysis, the five most prevalent genotypes worldwide are HPV 16 (3.2%), HPV 18 (1.4%), HPV 52 (0.9%), HPV 31 (0.8%), and HPV 58 (0.7%) [160]. Prevalence rates of the remaining genotypes are 0.6% or less, including for HPV 6 (0.5%) and HPV 11 (0.2%) (the two most prevalent genotypes associated with genital warts).

A different meta-analysis, including 260,000 women with normal cytology and 103,000 women with cervical abnormalities (including 36,000 with invasive cancer), reported an HPV prevalence rate of 12.0% among women with negative cytology [169]. This study also showed that

the prevalence of the HPV infection increases with the severity of cervical lesions, until about 90.0% HPV DNA-positivity among women with CIN3. As expected, apart from being the most prevalent HPV genotype worldwide, HPV 16 prevalence greatly increases with the severity of cytological and histological findings. The three most commonly found HPV genotypes in women with ICC (HPV 16, 18 and 45), were detected in a distinct proportion in women with normal cytology, with 20.0%, 8.0% and 5.0%, respectively, when compared to 63.0%, 16.0% and 5.0% in women with ICC, respectively (Fig.4) [169].

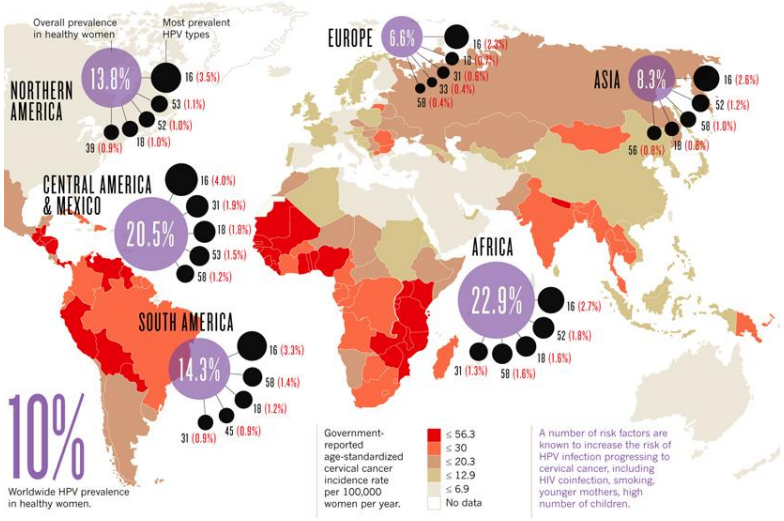


Fig. 4: Global cervical cancer incidence rate and HPV prevalence in asymptomatic women [www.nature.com].

It is somewhat difficult to compare the HPV genotype-specific distribution levels associated to cervical cancer development between different studies because of different inclusion criteria of study subjects and also due to different detection methods [170]. Considering the lifetime risk of women for acquiring HPV (80.0%), the most prevalent genotypes found in cervical cancer are HPV 16 (61.0%), followed by HPV 18 (10.0%), HPV 45 (9.0%), HPV 31 (6.0%), and HPV 33 (3.0%) [13,151,171]. Infections with multiple genotypes are fairly common as several population-based studies have reported that more than 30.0% of HPV-positive women are infected with more than one HPV genotype [161,167,172].

A higher HPV prevalence amongst young women might be due to different sexual behavior. The prevalence peak in women under 24 years coincides with sexual debut, with increasing risk for an early age at sexual initiation. Several studies have found that increasing numbers of lifetime sexual partners and numbers of sexual contacts (and consequently the number of sexual partners and contacts of the male partner) during the last year is associated with HPV 16 infection [157,167,173,174]. Considering that genital and perineal skin constitute reservoirs for HPV infection, condoms, if used incorrectly, only offer partial protection [175]. No association between condom use and protection from HPV infection was found in population-based cross-sectional studies performed by IARC [176]; however, a prospective study reported a 70.0% reduction on HPV incidence among

women whose partners “used condoms all the time”, compared to women whose partners “used condoms less than 5.0% of the time” [177]. HPV prevalence is higher in immunocompromised hosts such as renal transplant recipients and HIV-positive subjects [178]. Other factors that have been reported to increase the risk for HPV infection are smoking and oral contraceptives [157,179]. However, these factors tend to covariate with sexual risk-taking behavior and some studies have failed to find an association [177,180].

The potential cofactors involved in the progression from HPV infection to high-grade neoplasia, and subsequently invasion to cervical cancer, could be classified as virological, environmental and host factors. Virological cofactors includes HPV genotype, HPV variants, multiple HPV genotypes, viral load at the time of infection, and viral integration, while environmental factors includes use of hormonal contraceptives for a long time [180,181], high parity, tobacco smoking, and co-infection with other STIs, namely HSV2, *Chlamydia trachomatis* [180], and HIV [182].

Natural History of Cervical Cancer

Several prospective studies have provided a reasonably complete scenario of the natural history of the HPV cervical infection that leads to CIN3, but there is a poorer insight regarding the final steps of carcinogenesis (Fig.5) [137,183]. The moment of acquisition of the HPV infection seems to be the most difficult event to assess, so the majority of prospective studies based on cross-sectional populations may mixture short and long-term infections. To this point, a definition of persistence has not been established since different studies use distinct, arbitrary cut points. It is thought that HPV 16 has the ability to persist longer than other HPV genotypes [184-187] even with no development of CIN3 or worse (CIN3+). Moreover, it seems clear that the viral genotype constitutes the major risk predictor for CIN3+ development [64].

Of the estimated 291 million women infected by HR-HPV, the vast majority will become transient or develop persistently infected without any visible clinical symptoms (80.0%). The remaining will develop CIN, which may or may not progress to cancer [5,152]. Amongst the different cervical abnormalities that may be developed during an HPV infection, mostly will regress spontaneously, with subsequent clearance of HPV infection. Although the observed decrease of regression rate with increasing severity of CIN, cervical cancer represents a rare complication as it only affects nearly 2.0% of the HPV-infected women worldwide, and it occurs throughout a series of key events that take more than 10 to 15 years to unfold [188].

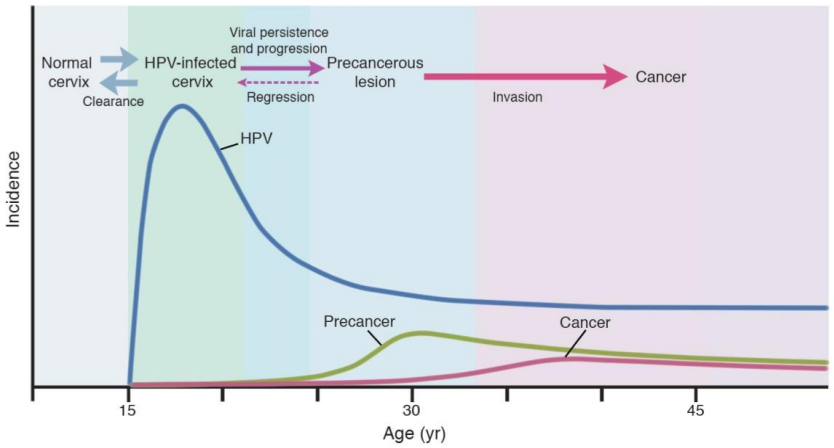


Fig. 5: Evolution of HPV infection and cervical cancer development [189].

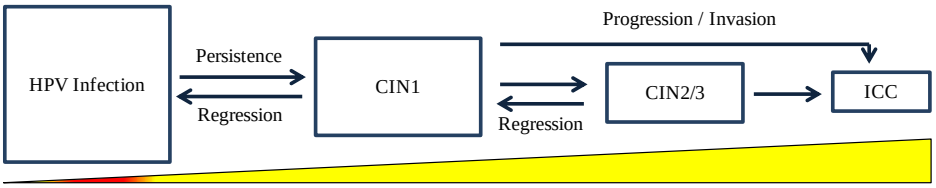
The natural history of cervical cancer develops through premalignant lesions. These lesions are classified as CIN and are detectable by cervical cytology and/or histopathology. In most of the European countries, squamous lesions are classified as CIN1, 2 or 3 representing increased severity of lesion [190]. In the USA, lesions are classified according to the Bethesda system where low-grade squamous intraepithelial lesion (LSIL) is equivalent to CIN1 and high-grade squamous intraepithelial lesions (HSIL) includes both CIN2 and 3 [191]. The histological classification of premalignant cervical glandular lesions is less clear but the WHO classifies lesions as glandular dysplasia and adenocarcinoma *in situ* [192].

Despite standardized criteria, it has been shown that there is substantial inter-observer variability in interpretation of CIN especially regarding CIN1 lesions [193] and CIN2 lesions. Recent data evidenced that regression rates of CIN2 among women under 25 years are quite high, reaching up to 70.0% in women aged under 24 years (with HPV 16-associated CIN2 regression close to 50.0%) [194]. In older women the regression rates are lower – around 30.0 to 50.0% through a two-year period [195,196].

The progression from CIN to cervical cancer is generally estimated in 5 to 10 years [5,197]. However, not all premalignant lesions will progress into cervical cancer. Estimates of progression and regression rates of different histological stages are highly uncertain, primarily due to misclassification of lesions and treatment of CIN lesions. Studies examining CIN progression rates have found that the risk is related to the severity of dysplasia (Fig.6) [198,199].

Women with cervical cancer were 15 times more likely to have had a previous Papanicolau (Pap) smear positive for HPV DNA, and a perfect concordance was observed between the HPV genotype detected in the Pap smear and the genotype detected in the subsequent cervical cancer. Several prospective cohort studies have found that particular HPV genotypes confer an increased risk for future premalignant lesions, with HPV 16 consistently inferring a very high-risk, suggesting that the most important viral characteristics linked to progression to cervical cancer, are the viral genotype

and its associated persistence rates rather than progression through distinct stages of cervical lesions [155,162,185,200-203].



	CIN1	CIN2	CIN3
Regression	57%	43%	32%
Persistence	32%	35%	56%
Progression	11%	22%	>12%
Invasion	1%	5%	

Fig. 6: Model of cervical carcinogenesis, where major steps are represented: HPV infection (balanced by viral clearance), progression to CIN (partly offset by regression of low-grade lesions), and invasion. The persistence, and ultimately the integration, of oncogenic HPV genotypes are necessary for lesion progression and tissue invasion [198,204].

Cervical Cancer Prevention

HPV primary prevention aims to avoid the infection and it relies on prophylactic vaccination. Secondary prevention attempts to reduce morbidity in pre-symptomatic subjects who already have established HPV infection and it is based on early detection and treatment, like screening programs to detect CIN and cervical cancer [205,206]. Tertiary cervical cancer prevention is to be applied in patients that already have clinical signs of the disease in view of cure, palliation, rehabilitation, or prevention of recurrence complications [205]. Preventive measures can be evaluated in terms of efficacy, defined as the extent to which an intervention produces a beneficial result in laboratory studies, or in terms of effectiveness, defined as the extent to how well that specific intervention does what it is intended for the population in clinical practice, when used under routine settings [207]. The natural history of cervical cancer makes it somewhat difficult to evaluate the efficacy of primary and secondary prevention, which is best assessed in randomized controlled trials while ensuring high internal validation [208,209].

Primary Prevention through HPV Vaccination

Two HPV vaccines are currently available, identified as Gardasil® (Merck & Co., Inc., Whitehouse Station, NJ, USA), often referred to as the quadrivalent vaccine and Cervarix®

(GlaxoSmithKline Biologicals, Rixensart, Belgium), also referred to as bivalent vaccine. Gardasil® targets the oncogenic HPV 16 and 18 genotypes and two non-oncogenic HPV genotypes (6 and 11) that are responsible for genital warts and RRP. Cervarix® only targets for the same two oncogenic HPV genotypes (16 and 18) and is formulated with a novel adjuvant, ASO4, intended to boost the immune response. At present, one or even both vaccines are licensed in approximately 120 countries worldwide, mainly included in routine vaccination programs targeting pre-adolescent and adolescent girls plus "catch-up" immunization in older cohorts with variable upper age limits [210,211].

To date, these two vaccines evidenced very high levels of efficacy against the CIN2+ disease endpoint defined for the oncogenic HPV 16 and 18 genotypes. So far, vaccination trials have shown adequate safety and tolerability, high immunogenicity, long-term duration of protection (ongoing follow-up of phase II studies have shown vaccine efficacy up to 8.4 years), and strong ability to induce immune memory [76]. It has been shown that these two vaccines confer some degree of cross-protection against CIN2+ lesions associated to other HR-HPV genotypes phylogenetically related but not included in the vaccines (HPV 31 for both vaccines and HPV 33 and 45 for Cervarix®). Therefore, the global estimates for the protection against cervical cancer can be increased up to 80.0% of cases, accounting for the HPV genotype cross-protection. However, the duration of cross-protection has not yet been determined and may well be less than for the HPV genotypes included in the vaccine. Protection for premalignant lesions of the vulva, vagina and anus has also been documented with the same vaccines [212,213]. Similarly, Gardasil® promoted almost complete protection (> 95.0%) against genital warts in both men and women [214,215]. Finally, HPV vaccines are putatively valid worldwide as HPV genotype-specific cervical cancer estimates show little geographical variation.

HPV vaccines lack therapeutic effects and thus require continuous screening programs, including among vaccinated women. Although the high cost of the vaccine has delayed its implementation, at present, public tenders reduced the cost, making vaccination and screening programs more affordable for developed countries [210,216]. HPV vaccination programs should induce substantial modifications over screening algorithms in a near future. First, vaccinated women will reduce the incidence of HPV 16 and 18-related precancerous lesions, decreasing the validity of screening test results, especially on cytology-based programs [217]. Furthermore, if second-generation broad spectrum vaccines become available, protection against cervical cancer might achieve 90.0 to 95.0%, dramatically reducing the incidence of the disease worldwide, so that screening might no longer be cost-effective in immunized cohorts [218]. The results of a nanovalent HPV vaccine trial will be soon available and might again change future perspectives for cervical cancer prevention [211,219].

Recent vaccine trials in men have shown their ability to prevent external genital warts, anal and penile HPV infections, as well as its associated lesions in MSM [213,215]. There are several major arguments favoring male vaccination for reducing cervical cancer: i) the expected impact on

herd immunity, whenever the vaccination coverage in women is lower than 70.0%; ii) the high impact on genital warts in men, considering the quadrivalent vaccine; iii) the reduction of HPV-related cancers in men genitalia; and iv) reduction of social concerns about safety of HPV vaccination, raised by promotion of single gender vaccination only. Alternatively, the major objections for male vaccination are: i) the late age-related burden of HPV-associated cancers in men (as opposed to early focus on cervical cancer in women); ii) the still limited data on potential impact of HPV vaccines over men's health; and iii) the high cost of vaccines that could imply that male vaccination is not cost-effective. Nonetheless, while women vaccination has been widely recommended signalling the public health priority for cervical cancer prevention, licensing of HPV vaccines for men has just been released in a few developed countries, so that some high-risk groups (MSM) are currently being vaccinated [220].

Cervical Cancer Screening[3]

Since the development of cytology-based cervical cancer screening, Pap smears and the new cytology-based technologies such as liquid-based cytology (LBC) have been implemented for prevention of cervical cancer. The advent of molecular techniques in HPV DNA detection evidenced cytological and histological diagnosis as not enough sensitive to predict HPV infection; in fact, no cytological or histological correlation of HPV infection can be detected in the majority of women who are positive for HPV DNA [168,221,222].

Cytological reading of cervical smears has been the primary screening technology, but due to test limitations, very frequent re-screening events were required to reach a cervical cancer reduction in the range of the 50.0% to 70.0% in the best settings (meta-analysis average) [223]. Indeed, cytology is a subjective labour intensive test and in the absence of quality control programs, it is virtually impossible to achieve and maintain the clinical necessary standards, due to low reproducibility and sensitivity of the Pap smear [224,225]. LBC has logistical and operational advantages, but is more expensive without any sensitivity or specificity increase comparing to conventional cytology for detection of histologically confirmed CIN2+ lesions [168].

HPV DNA testing, as an adjunct to cytology, may be of value in triage[4] of atypical squamous cells of undetermined significance (ASC-US) and LSIL, considering that only 10.0% of the women with abnormal smears have a premalignant lesion and colposcopy referral is not necessary for the majority of the HPV-infected women [226]. Triage with HPV DNA testing can help to identify women at higher risk and consequently improve their management [227-231]. In addition, HPV DNA

[3] The Portuguese cervical cancer screening programs will be described in Chapter 2, due to the importance of epidemiologic data for the objectives aimed in this PhD thesis.
[4] Term applied to selection of women with abnormal cytology using HPV DNA test for better risk evaluation in a screening program.

testing can be useful to predict cure or failure of clinical treatment (by local ablative or excisional therapy) in the follow-up of high-grade CIN lesions. The use of combined screening through cytology and HPV DNA testing should guide to a better selection of women at risk for residual/recurrent CIN, as suggested by some studies [231-234]. This combined procedure might avoid unnecessary procedures in patients without this risk factor, with important reduction of health costs and associated anxiety among women.

The persistence of detectable HPV DNA in CIN lesions prompted the evaluation of DNA assays as a screening tool for the early detection of CIN2+. All the findings evidenced an increase in sensitivity in the range of 35.0 to 40.0%, related with the use of HPV DNA tests when compared with conventional cytology or LBC. The corresponding decrease of specificity was in the range of 8.0 to 12.0% when compared to conventional Pap smears [223,235]. One HPV DNA test that could be used for screening in women with ASC-US is Hybrid Capture® 2 High-Risk HPV DNA Test (HC2) (Qiagen, Gaithersburg, MD, USA) [236], which has been widely implemented. Data obtained from meta-analysis reveals a better performance of HC2 to triage women with ASC-US, higher sensitivity and similar specificity, compared to repeated cytology-based re-screening testing [237]. Moreover, HC2 is considered significantly more sensitive for detecting CIN2+, favoring its recommendation to triage women who need referral to colposcopy [231].

New guidelines propose that an HPV DNA test should achieve clinical validation, and a clinical sensitivity of at least 90.0% for detecting CIN2+, in order to consider its recommendation for triage of abnormal smears [238]. However, this high clinical sensitivity usually means a lower specificity, around 40.0 to 60.0% (and the respective positive predictive value (PPV) of 20.0-30.0%), which is below optimal, requiring the need for using other specific markers such as HPV RNA testing. The management of women infected with HPV, and positive for secondary markers, is indicative of higher risk for cervical premalignant lesions and implicates more intensive follow-up than being negative for such markers. A recent meta-analysis has revealed that HPV RNA testing provides higher specificity but less sensitivity, when compared to HPV DNA testing, suggesting that these assays should be considered as reflex tests [231,239].

When considering triage of women with LSIL, HC2 test is not recommended, because is not more sensitive (and substantially less specific) than repeated cytological screening [235,240,241]. Management of women with LSIL requires knowledge on local prevalence of HPV infection and cost-effectiveness analyses, considering the high proportion of HR-HPV DNA in women with low-grade lesions. Women usually undergo colposcopy and if the biopsy reveals to be negative or CIN1, it is recommended an HPV test after 12 months or repeated cytology at 6-month interval. However, one can wait for viral clearance, especially in younger women, given the high HPV prevalence and the high regression rate that would reduce the need for colposcopy [134,240,242]. Restrict HPV DNA triage testing only to older women could be an alternative management option, but more accurate age-specific data is needed before defining exact age cut-off.

HPV DNA testing could be suitable for primary screening, but there is no sufficient data to corroborate that the actual cervical cancer incidence might decrease in accordance, more than what it would when cytology-based screening was used, mainly because some high-grade lesions might also regress. For this reason, HPV DNA-based cervical cancer screening programs have not yet been recommended in Europe [243]. Thus, HPV DNA detection is mainly advantageous for women older than 30 years, in the absence of more specific tests. If HPV DNA-based screening programs are implemented at a later age (since the majority of transient infections affect younger women), together with stretching the re-screening intervals (due to the higher sensitivity of these tests), and the eventual use of self-collected samples protocols (in low-resource countries), the efficiency of screening may increase [225,244].

The high negative predictive value (NPV) of HPV DNA tests, both for screening and for post-treatment of high-grade lesions ('test of cure'), have lead to new clinical procedures changing algorithms towards less frequent re-screening visits [233,245]. Low-cost HPV tests have started to be implemented in developing countries and should have a significant impact on mortality reduction in some settings [246-248]. Considering that they can be applied on self-collected samples, HPV DNA-based screening could reach socio-cultural environments where conventional cytology is not well accepted [244,249].

Some data [103,153] seem to show that the incidence of cervical cancer could be decreasing at a similar rate worldwide, as a result of the introduction of effective population-based screening procedures combined with the generalized easier access to health care and improvements in education [250].

New Screening Technologies

In this section is described the most promising of the novel screening markers for cervical cancer risk.

HPV DNA Tests

A single positive HPV DNA test predicts a higher risk for the development of a premalignant lesion, despite the expected clearance of the majority of infections [251]. HPV genotyping assays may differentiate sequential infection caused by different genotypes from a persistent infection with the same genotype, which would increase specificity of a routine screening program (Fig.7) [183,240,252,253]. Genotype-specific DNA positivity, especially for HPV 16 and 18 is highly linked to an elevated risk for development of cervical cancer, now extended to the risk of adenocarcinoma, which often escapes cytological screening [254]. The potential benefit in discriminating HPV 16 and 18 from other high-risk genotypes is the potential increase of the PPV, since these genotypes are

responsible for the majority of cervical cancer cases worldwide, which could provide better management of the women at risk [255]. In a cytology-based screening program, genotyping of HPV 16 and 18 from other HR-HPV genotypes may be important to stratify the risk for CIN3+ development, with an estimated cumulative risk of 17.0% for HPV 16, 14.0% for HPV 18, and 3.0% for other HR-HPV genotype [201,256-259].

Several international practice guidelines have recommended the use of HPV DNA testing in routine screening. So far, two commercial HR-HPV DNA-based tests have the approval to be used in screening from the Food and Drug Administration (FDA): the HC2 test (since April 2003) and the Cervista™ HPV HR assay (Hologic, Inc, Marlborough, MA, USA) (since April 2009) [255]. Both methodologies detect concurrently 13 HR-HPV genotypes (HPV 16, 18, 31, 33, 45, 51, 52, 56, 58, 59, and 68); Cervista™ HPV HR assay further includes HPV 66. Other commercial tests consist in a pooled detection of HR-HPV genotypes: the Amplicor HPV test (Roche Molecular Systems, Inc, Pleasanton, CA, USA), based in PCR amplification, and the Care HPV test (Qiagen, Gaithersburg, MD, USA), based on hybrid capture technology (suitable for low-resources settings) [255].

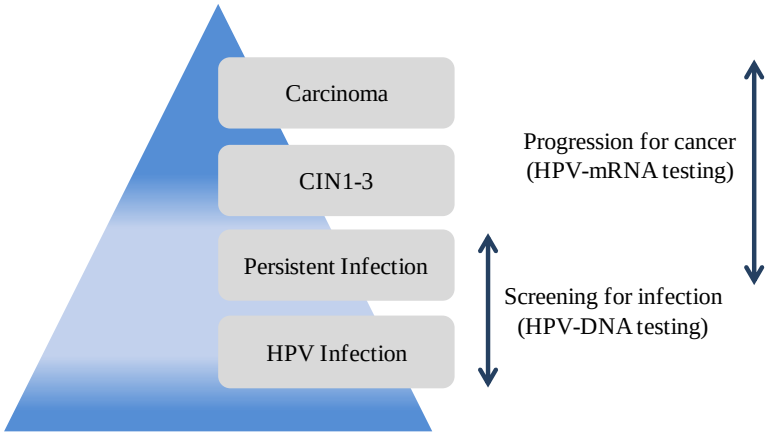


Fig. 7: Scheme of targeted spectrum of HPV infection/cervical disease cases in a screening population.

The clinical importance attributed to HPV 16 and 18 infections raised the emergence of HPV screening tests that include the specific identification of these genotypes, which are referred to as HR-HPV DNA detection assays with concurrent or reflex HPV 16 and HPV 18 genotyping [255]. There are four commercial assays that include concurrent or reflex HPV 16 and HPV 18 genotyping, two of them under FDA approval: the Cobas® 4800 HPV (Roche Molecular Systems, Inc, Pleasanton, CA, USA) (April 2011) and the Cervista HPV-16/18 test (Hologic, Inc, Marlborough, MA, USA) (April 2009). The other two are the Abbott RealTi*m*e High-Risk (HR) HPV test (Abbott Molecular, Des Plaines, IL, USA) and the HR HPV 16/18/45 Probe Set test (Qiagen, Gaithersburg, MD; USA) [255]. Cobas® 4800 HPV and Abbott RealTi*m*e HR HPV tests are fully automated systems based in multiplex real-time PCR methodology that identifies HPV 16 and 18 with concurrent detection of twelve other HPV genotypes (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Cervista HPV-16/18 test uses a patented Invader® chemistry to identify HPV 16 and 18 and

can be performed either manually or fully automated. HR HPV 16/18/45 Probe Set test consists in signal amplification based on hybrid capture technology using a specific probe designed for HPV 16/18/45 detection [255].

HPV genotyping tests are commonly used in studies regarding HPV genotype-specific prevalence, HPV vaccines evaluation and monitorization. The specific identification of HPV 16 and 18 is intended for screening programs. Nevertheless, each commercial assay should take part in international proficiency studies in order to assess their performance and potential clinical use in detecting precancerous lesions [260-262]. Several studies were published comparing HPV genotyping tests with HR-HPV DNA screening tests with reflex HPV 16 and HPV 18 genotyping and there seems to be some variability concerning their analytical sensitivity [263-265].

Concerning HPV genotyping systems, there are several commercial assays available, partially automated, that are either based on reverse-line blot hybridization: the INNO-LiPA HPV Genotyping Extra (Innogenetics, Gent, Belgium) (28 HPV genotypes) and the Linear Array HPV Genotyping Test (Roche Molecular Systems, Inc, Pleasanton, CA, USA) (37 HPV genotypes); or based on PCR-based microarray for genotyping: the PapilloCheck® Test Kit (Greiner Bio-One GmbH, Frickenhausen, Germany) (24 HPV genotypes); the CLART® Papillomavirus Humano 2 (Genomica, Madrid, Spain) (35 HPV genotypes); and the Infiniti HPV Genotyping Assay (AutoGenomics Inc, CA, USA) (26 HPV genotypes) [16,255].

Other commercial systems could serve as triage for HPV DNA-positive women, but there is still insufficient data. For instance, to use RNA testing in HR-HPV DNA-positive women, with cytology-negative and/or HPV 16/18-negative could increase specificity in detecting high-grade lesions with minimal loss of sensitivity [266].

It is anticipated that, since the implementation of HPV vaccines, the use of HPV genotyping in post-vaccination screening and/or surveillance studies is required. Population-based genotyping characterization pre- and post-vaccination should be important to determine overall vaccine effectiveness and potential unmasking of niche replacements by non-vaccine HPV genotypes.

HPV Viral Load

High viral load for most HR-HPV genotypes is associated with prevalent cervical cancer precursors [267]. HPV 16 viral loads have been associated with CIN2+ in cross-sectional studies and with progression to CIN2+ or ICC in prospective studies [268,269]. In fact, the association of high viral load with CIN2+ lesions seems to be true for most oncogenic genotypes; however, the prospective increased risk for CIN2+ associated with high viral loads is independent upon the genotype detected, and may be restricted to HPV 16 [270]. Translation of these observational viral load and disease associations into the clinical management of CIN is limited, due to the complexity of

the necessary evaluation of the assays, specimen sampling variability, and the common presence of more than one carcinogenic genotype.

At present, the few commercial methodologies available do not provide exact viral load quantitation data, but the HC2 test is a semi-quantitative methodology for detection of HPV DNA. Other methods for viral load quantitation include both conventional and real-time polymerase chain reaction (PCR). The obvious limitation of the real-time PCR method is the requirement for multiple genotype-specific assays. Yet, quantitative assays of HPV genotype-specific viral load lack prognostic value, and require cost-efficacy studies on genotype-specific quantitation testing compared to other screening approaches.

HPV Integration

HPV 16 integration is commonly detected in CIN2+. Multiple methods for detection of integrated HPV DNA have been described [271-273]; however, most of them evidenced limitations similar to those described for HPV viral load quantitation. Because cervical epithelial cells from women with CIN1 or CIN2+ may simultaneously contain episomal and integrated HPV DNA, the use of integrate-specific DNA PCR methods could be misleading and induce misclassification of cases [274]. Differentiation of transcriptionally active integrants could, in theory, constitute a more specific marker of disease risk; however, at present, the assays are too complex to be used for routine purposes. In addition, recent data suggest that integration frequency among pre- and malignant lesions varies with the HPV genotype involved, further reducing the desired gains in specificity [275]. A comparative study between integrated forms of HPV 16 and 18 DNA in CIN development showed a trend in the prognostic value of this biomarker for HPV 18 infection cases, related with a more aggressive biological behavior of this genotype [276].

It is unlikely that a single screening test will provide the necessary clinical sensitivity, thus, some false negatives are to be expected. An increment in the clinical specificity or the PPV is the goal of future screening tests. The identification of potential biomarkers with predictive value for cervical neoplastic progression remains an important priority, following future adaptation in screening programs.

HPV mRNA

The oncoproteins E6 and E7 play a leading role in the carcinogenesis process, as these proteins are overexpressed throughout epithelial cells in high-grade lesions and cancer. This means that overexpression of E6 and E7 would distinguish transient infections from productive, inductive of malignant changes, infections. Thus, detection and quantitation of mRNA from E6 and E7 genes of HR-HPV could be a more specific marker of the presence of CIN2+ and ICC.

Commercial tests targeting HPV mRNA are now available: APTIMA® HPV (Hologic Gen-Probe Incorporated, San Diego, CA, USA) and NucliSENS® EasyQ® HPV (bioMérieux SA, Marcy l'Etoile, France). These assays present different approaches, meaning that they should be used at different stages of the screening algorithm. The APTIMA® HPV assay does not distinguish the HR-HPV genotype; in fact, it can qualitatively detect the presence of mRNA of at least one of 14 HR-HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and is already approved by the FDA. The approach used in this assay, while increasing the specificity and the PPV, will also achieve sensitivities and NPVs similar to HPV DNA tests, recommending its possible use in routine screening as co-testing with cytology [223,277,278]. But working with mRNA represents higher technical complexity related with the low RNA stability, which resolution would increase costs, and more importantly, the compatibility with the different sample collection buffers is unknown. NucliSENS® EasyQ® HPV only semi-quantifies the overexpression of E6 and E7 genes for five HR-HPV genotypes (HPV 16, 18, 31, 33, and 45), the most associated with cervical cancer cases. The higher specificity of this mRNA assay would reduce false positives, as they would exclude transient infections, so they could be used as a triage test for HPV DNA-positive women, reducing the colposcopy referral in cytological abnormalities and therefore improving patient management [233,279].

OBJECTIVES

Worldwide, cervical cancer is a major public health problem, and there is irrefutable evidence that cervical infection by oncogenic (high-risk) HPV genotypes is the primary risk factor for the development of this neoplasia.

Despite the introduction of HPV vaccines in several developed countries, the expected reduction on HPV infection rate among women, and the reduction in cervical cancer incidence rates are yet to be observed, mostly because, when the vaccine was introduced, a vast majority of women was already infected by HPV. Also, considering the discrepancy between HPV vaccination age and cervical cancer screening age, several decades will be needed before considering any slowdown on cervical cancer screening programs.

Nowadays, cervical cancer prevention through organized screening programs is essential for the management of HPV-infected women. Conventional cytology, highly specific, has long been considered as the standard cervical cancer screening method; however, it lacks sensitivity for detecting the early stages of the disease. More recently, molecular biology research boosted the sensitivity of HPV molecular diagnostic tests, but the later recognizably lack specificity and, more importantly, they lack clinical validation to predict disease, which is essential for its recommendation for cervical cancer screening (even when used together with cytology). So, which is the best strategy for cervical cancer screening in a near future? The answer relies on country economical conditions and population characteristics (age distribution, sexual habits), and on local HPV epidemiological data (prevalence, HPV genotype-specific distribution) which, together, would determine the optimal management of HPV-infected women.

From the exposed and considering the impact of cervical cancer in Portugal (one of the highest rates in Europe), the general goal of the present PhD thesis was to better understand HPV biology and carcinogenesis by evaluating different biomarkers for cervical disease. Particularly, several HPV DNA tests (in what concerns sensitivity and specificity, PPV and NPV, and adequacy for screening in each disease stage) were evaluated as well as some recognized molecular markers (HPV genotype, viral load, viral integration), either during opportunistic and selected screening (involving women with and without cervical lesions). Cervical cancer screening in Portugal was analyzed and alternative strategies and future scenarios were proposed, the later according to the mathematical models developed during the present study.

In detail, the following objectives were pursued, constituting the subject of each chapter of the present PhD thesis:

To further the understanding of HPV infection in women, in order to develop cancer prevention strategies applied to local settings (**Main goal 1**):

- To assess the overall prevalence of HPV in cytological normal smears, ASCUS, LSIL, HSIL, CIN, ICC and other glandular lesions (including adenocarcinoma) in the general female population of selected areas in Portugal;
- To determine HPV infection age-specific distribution;
- To determine oncogenic HPV genotype-specific distribution;
- To determine HPV-specific patterns concerning single and multiple infections;
- To perform clinical validation of HPV DNA assays.

As a way to fulfil this main goal, 2149 samples were selected from women attending to Human Papillomavirus Reference Laboratory at National Institute of Health (INSA) for HPV testing (opportunistic screening), and further associated with the respective clinical diagnosis and age anonymously. This global evaluation constituted a prospective epidemiological study, where it was evaluated risk factors for HPV infection and cervical cancer. In Chapter 3, the selection criteria were exposed in detail, as well as the laboratory procedures performed to assess the overall HPV prevalence and HPV genotype-specific distribution. A new selection of 731 samples of women with and without cervical disease (within the primary 2149 samples) constituted the study group to evaluate the clinical performance of different HPV tests for further use in clinical practice (Chapter 5). A control group was constituted by samples from women who had not developed disease at the time of HPV diagnosis. An extensively detailed evaluation and clinical validation of one commercial system HPV DNA test was the subject of Paper II (Chapter 4).

To evaluate the importance of molecular markers for early diagnosis of women at risk for HPV persistence and lesion progression (**Main goal 2**):

- To establish the clinical utility of a commercial system of E6/E7 mRNA assay in routine screening/triage of normal, ASCUS and low-risk lesions by predicting which HPV-positive women will develop pre-malignant or malignant lesions;
- To assess viral load as a predictive molecular marker of persistent high-risk infection;
- To assess physical status of HPV DNA as a molecular marker for disease progression.

The detailed evaluation of the clinical performance of one mRNA commercial system was the subject of Paper III (Chapter 6). The evaluation of potential molecular markers (viral load and

physical status assessment) was performed on a subset of 499 samples that were previously genotyped as HPV 16 and 18 within the primary 2149 samples. This evaluation relied on real-time PCR methodology, and each molecular marker was assessed in the same specimen (Chapters 7 and 8). In order to apply these methodologies to cytological cervical samples, while reducing the need for colposcopy referral, the same nucleic acids extraction was used to each individual molecular marker under evaluation. Further analysis on which methodology was more adequate to prevent HPV 16 and 18 high-grade cervical lesions development was discussed on Chapter 10.

To provide baseline information to evaluate future screening and vaccination strategies suitable for the population of the selected areas (**Main goal 3**):

- To apply mathematical models in the evaluation of the impact of HPV vaccination and to estimate the risk factors for HPV infection.

All data collected throughout the following chapters was used to formulate different scenarios applying a mathematical modelling approach which was discussed in detail in Chapter 9. A proposed screening strategy was presented in Chapter 10 that includes general discussion and future perspectives.

CHAPTER 2

Ana Gradíssimo, Nuno Verdasca

Contributions of the Authors:

Ana Gradíssimo was responsible for the research, writing and organization of this paper.

Nuno Verdasca contributed with proofreading, image rearrangements and compilation of information to be included.

ORIGINAL ARTICLE

Cervical Cancer Screening in Portugal: A Perspective

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ABSTRACT

Portugal has one of the highest incidence and mortality rates for cervical cancer in Europe, evidenced by the 949 new cases and 346 deaths registered in 2008. The CLEOPATRE Portugal Study has provided baseline epidemiological data, where primary prevention through human papillomavirus (HPV) vaccination appears to be a promising tool for preventing cervical cancer. Furthermore, that study showed that an appropriate screening strategy could reduce the current cervical cancer incidence in almost 50.0%.

Cervical cancer screening programs have been implemented regionally in Portugal, and the different algorithms may influence the expected reduction in cervical cancer incidence rates. The imbalanced geographical distribution of these programs can worsen the access to HPV opportunistic screening of low-resource populations that rely completely on the free of charge programs provided by the national healthcare system. HPV vaccination has been introduced in the national immunization program for girls aged 13 years, with an estimated coverage rate of 80.0%.

Worldwide, cervical cancer is an important public health problem, and the intention to reduce its incidence includes taking public health decisions and the design of strategies for prevention, screening and treatment. Preventive measures include health education to decrease risk behaviors, and, more recently, HPV vaccination. Screening aims to early detect and diagnose cytological abnormalities and precancerous lesions, in time to apply successful treatment options and ultimately prevent cervical cancer.

Keywords: Cervical cancer, screening programs, human papillomavirus, Portugal.

In Submission.

EPIDEMIOLOGICAL DATA

Incidence rates of cervical cancer across Europe are highly variable, with the lowest European incidence rate is observed in Finland (3.7 new cases per 100,000 women), while the reported incidence rate in Portugal is considerably higher (12.2 new cases per 100,000 women) [1]. A cross-sectional population-based study was conducted in mainland Portugal between 2008 and 2009, "The Cervical Lesions Observed by Papillomavirus Types – A Research in Europe (CLEOPATRE)" [2]. This study constitutes baseline information regarding prevalence of human papillomavirus (HPV) infection (overall and age-stratified) and comprises data from 2326 women selected from the five different regions across mainland Portugal.

The overall HPV prevalence observed in the CLEOPATRE study was 19.4% (451/2326; 95% CI: 17.8-21.0); the estimated prevalence of HPV infection among Portuguese women aged 18 to 64 years was 12.7% (95% CI: 11.2-14.4). It was observed a significant association of HPV infection with age, where young women (aged < 25 years) evidenced the highest prevalence rates, as expected from the described by other studies (Table 1) [2-5].

Table 1: Prevalence of HPV infection by age group in the CLEOPATRE Portugal Study sample and estimated prevalence (weighted) for the general female population of mainland Portugal aged 18 to 64 years

Age Group, Years	Study Sample				Female Population of Mainland Portugal		
	No. Women Tested	No. Women HPV-positive	% (95% CI)	P*	n	% (95% CI)	P*
Total	2326	451	19.4 (17.8-21.0)	<0.001	3,293,911.6	12.7 (11.2-14.4)	<0.001
18-19	274	74	27.0 (21.8-32.3)		106,974.1	26.4 (21.5-31.9)	
20-24	691	199	28.8 (25.4-32.3)		299,096.8	28.7 (25.5-32.2)	
25-29	458	100	21.8 (18.1-25.6)		365,669.5	21.8 (18.3-25.9)	
30-39	256	32	12.5 (8.5-16.6)		788,049.2	12.4 (8.9-17.1)	
40-49	223	22	9.9 (6.0-13.8)		755,443.1	9.6 (6.4-14.2)	
50-59	228	13	5.7 (2.7-8.7)		675,971.5	5.5 (3.2-9.2)	
60-64	196	11	5.6 (2.4-8.8)		302,707.4	5.7 (3.2-10.0)	

n, standardized female population of mainland Portugal (weighted value); CI, confidence interval; *Pearson χ^2 test. [2].

Among HPV-positive women, infection with high-risk HPV (HR-HPV) genotypes was identified in 76.5% (345/451) of the cases, and the five most common circulating HR genotypes were HPV 16 (19.7%), followed by HPV 31 (11.8%), HPV 53 (11.8%), HPV 51 (9.8%), and HPV 66 (8.6%), while HPV 18 was only detected in 4.4% of the infected women. This distribution pattern has been observed in several other European countries (Table 2) [6-11]. A statistically significant association could be established between the prevalence of HR-HPV infection and age ($P < 0.001$), and consequently HR genotypes were more frequent in younger women, in particular for the cohort aged 20 to 24 years (23.4%).

Multiple infections were identified in 7.1% (165/2326) of the studied women, and in 36.6% (165/451) of the HPV-positive cases (estimated prevalence, 3.7%). Multiple infections were more

frequent in younger women but less frequent in normal cytologies or high-grade cervical lesions [2]. Considering the genotypes included in HPV vaccines, HPV 16 was the most frequent (3.8%), whereas HPV 6 (1.2%), HPV 18 (0.9%), and HPV 11 (0.4%) were scarce. Infection with at least one of these genotypes constitutes 32.6% of the HPV-positive cases, and co-infection with two of these genotypes was identified in 2.0% of those.

Table 2: HPV DNA prevalence (%) in women with high-grade lesions by world region

World		Europe		Portugal	
HPV Type	**Prevalence**	**HPV Type**	**Prevalence**	**HPV Type**	**Prevalence**
16	45.4%	16	51.8%	16	49.7%
31	8.7%	31	10.0%	31	11.8%
33	7.3%	33	8.6%	58	11.2%
58	7.0%	18	6.0%	33	5.9%
18	6.9%	52	3.6%	51	5.9%
52	5.1%	73	3.5%	52	5.3%
35	3.8%	35	3.4%	18	4.7%
51	3.6%	51	3.0%	35	4.7%

The grey areas highlight HPV 16 and 18 prevalence rates. [2,12]

In this screening-type population, 93.8% of the women exhibited a normal cytology. Stratification according to cytology revealed that HPV infection was detected in 16.5% of the normal cytologies (NILM) (estimated prevalence, 12.1%), but increased severity of cervical lesions was associated with higher values of HPV detection, namely 21.6% in atypical squamous cells of undetermined significance (ASC-US), and 54.5% in low-grade squamous intraepithelial lesion (LSIL). A statistically significant association could be established between infection with HR-HPV genotypes and cytological abnormalities ($P < 0.001$) [2]. These results are similar to the described in North European countries [4,13], but higher than the expected, considering what was described for other neighboring and Mediterranean countries [10,14,15].

Risk Factors

CLEOPATRE Portugal Study also investigated potential risk factors for the acquisition of HPV infection. Women enrolled in the study reported using contraception (89.1%), a single lifetime sexual partner (57.3%), no prior sexual transmitted infections (STI) in the past 12 months (96.0%), were not immunocompromised (97.6%), and were currently smokers (22.3%) [16].

Young age increased the risk for HPV infection, with an odds ratio (OR) of 6.80 (95% CI: 0.87-13.90) for women aged 20 to 24 years. In multivariate analysis, women aged 20 to 24 years were 3-fold more at risk than women aged 60 to 64 years, as it was described in other studies [4,17,18]. Smoking was associated with an increased risk for HPV infection (OR 2.18; 95% CI: 1.73-2.75), while among current smokers, the risk of HPV infection increased with the number of cigarettes smoked. Age at first sexual intercourse (< 14 years) (OR 4.39; 95% CI: 1.37-14.13) and a high number of lifetime sexual partners (5-10 partners) (OR 8.58; 95% CI: 5.75-12.82) were both strongly associated with HPV infection. Having 5 to 10 sexual partners represented a 5-fold increased

risk for HPV infection (OR 5.44; 95% CI: 3.51-843), in multivariate analysis (P < 0.001), as described elsewhere [19]. Use of contraception increased the risk for HPV infection (OR 2.12; 95% CI: 1.40-3.20), where oral contraceptives induced the higher risk (OR 1.45; 95% CI: 1.04-2.03). Having a circumcised sexual partner represented an elevated risk (OR 1.72; 95% CI: 1.27-2.34), as opposed to women without STIs in the past 12 months (OR 0.31; 95% CI: 0.18-0.53), where the risk was minimal [16].

SCREENING PROGRAMS

Screening strategies imply taking decisions that would be cost-beneficial for reducing incidence and mortality rates of cervical cancer. For more than 50 years, cervical cytology (conventional Papanicolau (Pap) smear or liquid-based cytology - LBC) has been the standard test for screening [20]. In regions where cytology-based programs have been successfully implemented, a reduction of 50.0 to 90.0% in cervical cancer incidence rates has been observed [21]. There is now substantial and consistent evidence that HPV DNA testing is about 20.0 to 45.0% more sensitive in detecting precancerous lesions, when compared to cytology [22-28]. The use of a single HPV DNA test could effectively reduce the incidence of cervical cancer within 4 to 5 years [26,28], and the mortality rate within 9 years [29], when compared to the impact of the use of a single cytology test. This made possible the recommendation of HPV DNA testing to be used as an adjunct to cytology (in a co-testing strategy), or as the primary screening test that would be followed by cytology [21]. Also, HPV DNA testing may be used in self-collected samples which can be an additional advantage for their use in poor clinical settings such as developing countries. In fact, the proven high sensibility of HPV DNA testing in detecting high-grade cervical lesions (CIN2+) determined its recommendation as primary screening test, especially due to the low sensitivity of the Pap smear test. However, repeated HPV genotyping test is needed before the identification of a HR-HPV persistent infection, which is the major risk factor for cervical cancer development. Thus, the use of HPV DNA testing as a primary screening test is only recommended in women older than 30 years, considering that HPV infections in younger women are usually transient and, therefore, clinically irrelevant. HPV DNA testing would benefit from specificity increment that might become available in a near future, leading to the universal recommendation for the triage of women with cytological abnormalities [22,30,31].

Data from the CLEOPATRE Portugal Study suggested that vaccination could reduce HPV-related disease burden in Portugal. However, the expected reduction outcome is difficult to assess as screening policies are not equally implemented across the whole country. In fact, differences on baseline testing (cytology or HPV DNA test), age and triage testing, and management of HPV-positive women, differ among country regions. Since 2008, along with the introduction of HPV vaccines in the Portuguese national immunization program, different organized population-based

cervical cancer screening programs have been implemented by the five regional health administrations (ARS) (Fig.1). As it was pointed in 2012 in a report by the General Directorate of Health (DGS), the coverage rate is almost 100% in four of the five ARS, but for the remaining it is inexistent (ARS *Lisboa e Vale do Tejo*). This imbalanced geographical distribution can worsen the access to HPV opportunistic screening, especially for populations with low-resources that rely completely on the free of charge programs provided by the national healthcare system [32].

Fig. 1: Regional distribution of the five ARS in mainland Portugal [adapted from https://ec.europa.eu/].

ARS *Centro*

The first Portuguese screening program was implemented by the ARS *Centro* back in 1990, which was designed to screen sexually active women aged 25 to 64 years, involving 65 healthcare centers. In 2007 the geographical area was expanded to the entire region (109 healthcare centers). It is expected to cover 481,000 women, by providing a cytology-based screening (Pap smear) program, within a 3-year re-screening interval. The women are recruited by post mail from the database of the different healthcare centers. In 2012, this cervical cancer screening program comprehended about 84,000 cytologies, which corresponded to 58.0% of coverage rate (Fig.2) [33].

Cytological findings are classified according to the Bethesda System [34]. Histopathologic evaluations are performed in the two regional reference hospitals (Coimbra Portuguese Oncology Institute (IPO *Coimbra*) and Coimbra University Hospitals (HUC)) which apply quality control proceedings. For the recommended algorithm, each time a NILM result is obtained these women are conducted to re-screening. If an ASC-US result is identified, women are referred to a repeated cytological evaluation within six months, and if this test is negative women are conducted to re-screening; in opposition, a positive result will lead women to colposcopic evaluation. When a cytological diagnosis of LSIL or worse is obtained, women are referred to one of the two reference regional hospitals for colposcopic evaluation. If minor abnormalities are detected in the biopsy, cytology is to be repeated within six months; in opposition to detection of major abnormalities, in which excisional treatment of cervical lesions will be performed.

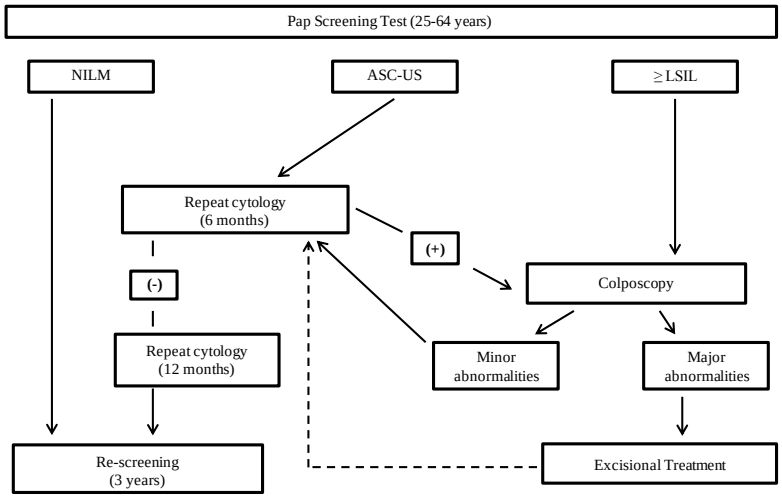

Fig. 2: Simplified screening algorithm of ARS *Centro* [33].

ARS *Alentejo*

In 2008 ARS *Alentejo* implemented a cervical cancer screening program that was designed to screen sexually active women aged 30 to 64 years, involving 44 healthcare centers. In 2011, the geographical area was expanded, and another four healthcare centers were included, while the age range was enlarged to 25 to 64 years. It is expected that this program covers 148,000 women, providing a cytology-based screening (LBC) program and an HPV genotyping triage test in women with ASC-US. The women are recruited by post mail according to the different healthcare centers database. For the period between 2008 and 2012, more than 70,000 cytologies were already performed and a coverage rate higher than 80.0% was achieved (Fig.3) [35].

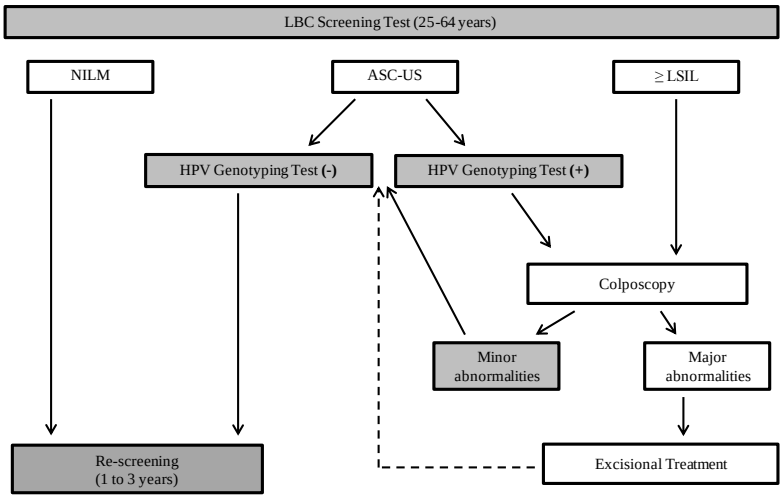

Fig. 3: Simplified screening algorithm of ARS *Alentejo* [35]. The grey areas correspond to changes in the algorithm, when compared to the algorithm of ARS *Centro* (Fig.2).

Cytological findings are classified according to the Bethesda System [34]. Both tests are performed in a central laboratory (Evora Central Hospital) which applies quality control proceedings.

For the recommended screening algorithm, it is intended to perform two LBC cytologies, one year apart, and if two negative cytology results are obtained, the re-screening interval increases to a 3-year interval. A negative result implies re-screening after 12 months; in opposition, if one of the cytological results reveals ASC-US, an HPV genotyping triage test will be performed, and if the later is also positive, women are referred to colposcopy in the central hospital. After colposcopy, if the biopsy evaluation reveals minor abnormalities, women are recruited to a new HPV genotyping test within 12 months. In the presence of major cervical abnormalities, excisional treatment options are undertaken, followed by an HPV genotyping test within 12 months. An HPV genotyping-negative result leads women back to re-screening; in opposition to an HPV genotyping-positive result that justifies a new referral to colposcopy. Women evidencing LSIL are referred to colposcopy and biopsy evaluation, and will undergo adequate procedures (according to the guidelines for ASC-US with an HPV-positive test). Women with atypical squamous cells that cannot exclude HSIL (ASC-H) lesions are referred to colposcopy and further biopsy evaluation, and will undergo the treatment options adequate to high-grade lesions. However, if the biopsy does not reveal cervical lesions, cytology is to be reviewed and followed by an HPV genotyping test within 12 months. Women with high-grade squamous intraepithelial lesions (HSIL) will undergo excisional treatment followed by an HPV genotyping test within 12 months.

ARS *Norte*

Up to 2009, opportunistic screening was covering about 40.0% of women living in the area covered by ARS *Norte*; however, women with low-resources were not involved in this system and consequently, they would be at higher risk for cervical cancer. With an estimated incidence rate for cervical cancer of 3.6 per 100,000 women, and 325 new cases of cervical cancer per year that resulted in 65 deaths, ARS *Norte* decided to implement an organized cytology-based screening (LBC) program, using Hybrid Capture® 2 HR HPV DNA Test (HC2) as triage test. Women are recruited by post mail, and 103 healthcare centers are currently involved, leading to an expected coverage rate above 70.0%. Age of women selected for screening ranges between 25 to 60 years with a 5-year re-screening interval (corresponding to a total of eight re-screening episodes during each woman lifetime) (Fig.4) [36].

When NILM cytology is detected, women are conducted to re-screening. In the presence of both ASC-US cytology and HPV-negative results, women are called for re-screening within 12 months. If an HPV HC2-positive outcome or a LSIL cytology result is observed, women are referred to colposcopy for a diagnostic evaluation. When minor abnormalities are observed a HC2 triage test is performed; in opposition, when major abnormalities are identified, an excisional treatment must be done. For an ASC-H/HSIL cytological diagnosis, women are recommended for excisional treatment through conization in the central Hospital (Oporto Portuguese Oncology Institute (IPO *Porto*)) of the ARS *Norte*.

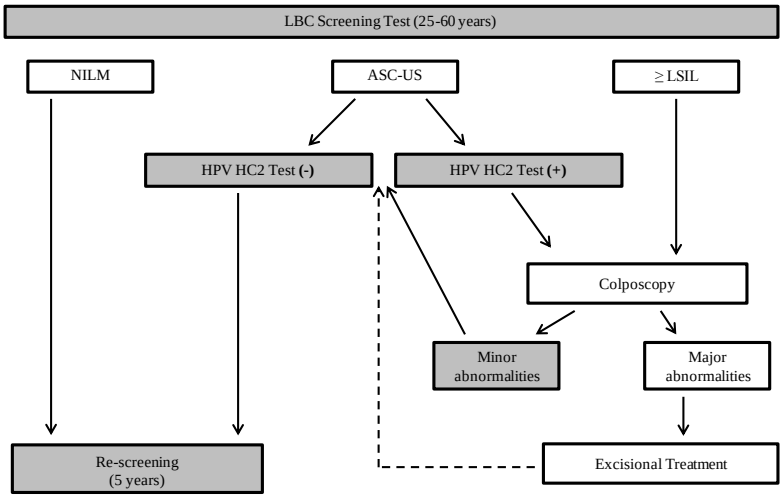

Fig. 4: Simplified screening algorithm of ARS *Norte* [36]. The grey areas correspond to changes in the algorithm, when compared to the algorithm of ARS *Centro* (Fig.2).

ARS *Algarve*

ARS *Algarve* implemented a cervical cancer screening pilot program in 2010. The estimated incidence and mortality rates for cervical cancer are the highest of the country, implying 15 new cases per 100,000 women and 6.8 deaths per 100,000 women, respectively. A cytology-based screening (LBC) program was implemented using HPV DNA testing as triage test, and a 3-year re-screening interval. Women are recruited by the healthcare centers through post mail, and it is expected to cover 141,425 women within an age range of 25 to 64 years (50,000 cytologies annually), corresponding to a coverage rate of 40.0% (Fig.5) [37].

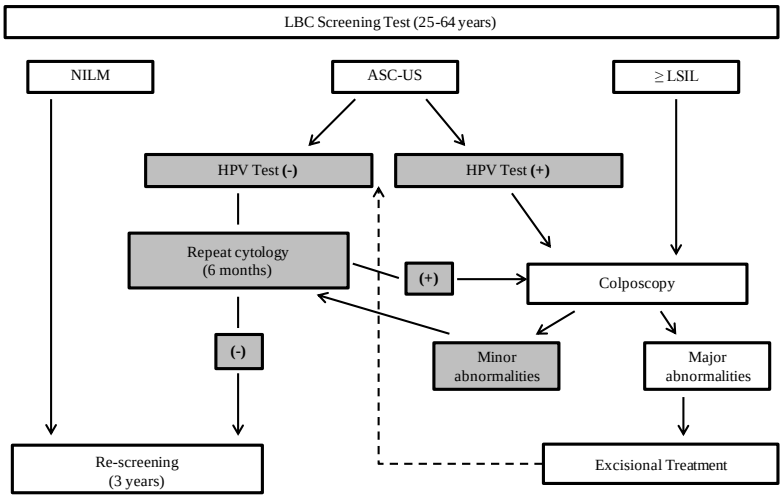

Fig. 5: Simplified screening algorithm of ARS *Algarve* [37]. The grey areas correspond to changes in the algorithm, when compared to the algorithm of ARS *Centro* (Fig.2).

If NILM cytology is detected on a first screening episode, women are conducted to re-screening. For an ASC-US cytological result, an HPV DNA test will be performed, and if the later comes negative, cytological evaluation will be repeated within six months; if this HPV DNA testing

is negative, women return to re-screening. For an ASC-US with an HPV DNA-positive test, women are referred to colposcopy at one of the two reference regional hospitals (Barlavento Algarvio Hospital (HBA) and Faro Hospital (HF)). In case of detection of minor abnormalities, women repeat cytological evaluation within six months; in opposition, when major abnormalities are detected, women will undergo excisional treatment followed by HPV DNA testing within 12 months. For a cytological evaluation of LSIL or worse (ASC-H, HSIL, cervical cancer) women are referred to colposcopy for biopsy evaluation; when biopsy reveals the presence of major abnormalities, women undergo excisional treatment. Since the implementation of the pilot screening program, 93.0% of the target population has already benefited from the first cytology. In 2013 until April, 20 women have been referred to colposcopy with a LSIL cytological diagnosis.

ARS *Lisboa e Vale do Tejo*

In 2005, ARS *Lisboa e Vale do Tejo* estimated the mortality rate associated to cervical cancer in 3.4 per 100,000, comprehending a peak of incidence and mortality for women aged 45 to 54 years. Guidelines are still under evaluation for a screening program that expects to involve 893,886 women aged 30 to 64 years to be screened (coverage rate of 60.0 to 80.0%), through a cytology-based screening (LBC) program every five years (> 160,000 cytologies per year) (Fig.6) [38].

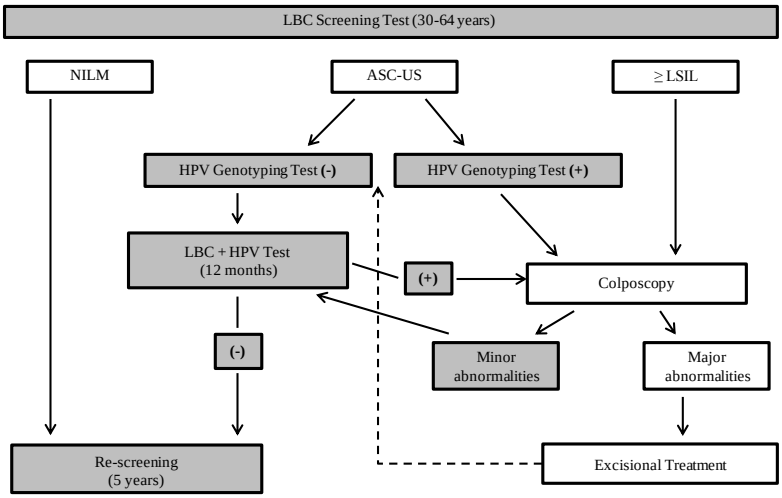

Fig. 6: Simplified screening algorithm proposed of ARS *Lisboa e Vale do Tejo* [38]. The grey areas correspond to changes in the algorithm, when compared to the algorithm of ARS *Centro* (Fig.2).

For ASC-US cytologies it is intended to perform an HPV genotyping triage test (as implemented by ARS *Alentejo*) to allow the discrimination of HPV 16 and 18 considering the specific association of these genotypes with precancerous lesions. This strategy will facilitate future evaluations of the HPV vaccination program, and will reduce cross-reactions often described for the HC2 test (5.0 to 13.0%); it will increase sensibility to CIN2+ detection, without a significant decrease in specificity [30,39,40]. HPV genotype detection among ASC-US cases is expected to be higher than 50.0%, so that more than 3500 colposcopies will be necessary. The estimated detection rate for

LSIL and HSIL lesions is 3.0% and 1.0%, respectively, corresponding to more than 6000 colposcopies. Also, almost 1000 cases for treatment and follow-up in the reference regional hospital in the region (Lisboa Portuguese Oncology Institute (IPO *Lisboa*)) are expected. This reference hospital will be collaborating with ten histopathologic laboratories and two HPV genotyping testing reference laboratories (IPO *Lisboa* and National Institute of Health (INSA)).

HPV VACCINATION PROGRAM

The quadrivalent HPV vaccine Gardasil® (Merck & Co., Inc., Whitehouse Station, NJ, USA) was commercialized for the first time in Portugal in December 2006 [41]; and from October 2007, the bivalent HPV vaccine Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) also became available [42]. Two studies supported the cost-effectiveness of HPV vaccine in Portugal as they showed that HPV vaccines would be effective [43,44]. These two HPV vaccines were approved by the Portuguese DGS for preventing HPV 6, 11, 16 and 18 infections (including persistent infections), and their associated lesions (cervical intraepithelial neoplasia (CIN) grade 1 to 3) and cervical cancer [45]. A reduction in the proportion of cytological abnormalities is expected to occur in a short term period, after the introduction of HPV vaccination, and, ultimately, a reduction in the number of cervical cancer cases is the main objective of this expensive prevention scheme [46].

The Portuguese immunization program for HPV was implemented in October 2008 for girls aged 13 years; from 2008 to 2011 girls aged 17 years were also given the opportunity to benefit from vaccination through a catch-up program. The Portuguese HPV vaccination scheme includes three doses which one provided free of charge by the national healthcare system, for covering more than 70.0% of the target population [45,47]. The decision of including the HPV vaccine in the Portuguese immunization program was mainly based on the estimated high cervical cancer incidence and mortality rates among Portuguese women, 12.2/100,000 and 3.6/100,000 women, respectively. The limited coverage of organized cervical cancer screening programs in mainland Portugal up to 2008 also contributed for the decision to vaccinate all young Portuguese girls [45,46].

Based on worldwide prevalence data, where the oncogenic HPV 16 and 18 genotypes are responsible for 70.0 to 75.0% of the cervical cancer cases, a reduction of about the same proportion is expected in Portugal [2]. The Portuguese Ministry of Health [http://www.portaldasaude.pt/portal], estimates that 80.0% of the 15 years old girls have been already vaccinated, which is near to the 85.0% coverage goal, but it can still increase considering that HPV vaccine intake is free of charge for women under 25 years which had already initiated HPV vaccination program [48]. Despite of the concern and efforts of the health authorities, population awareness on cervical cancer is limited, namely among university students, who evidence an obvious lack of knowledge on HPV transmission, HPV diagnosis and HPV consequences, which may affect the success of HPV

vaccination social acceptance [49]. So, improvement of education and screening strategies is needed for an effective prevention of HPV-related diseases in Portugal. Vaccinating boys against HPV has not been considered cost-effective in Portugal, considering the high coverage rate for adolescent girls [50].

DISCUSSION

Worldwide, cervical cancer screening programs are very different within countries that have already implemented one, despite the similar recommendations from international practice guidelines for Europe and United States of America (USA). The main differences are centered in the age range, time to re-screen, primary screening test (cytology versus HPV DNA test), type of testing (conventional versus LBC cytology; and HPV DNA versus HPV genotyping tests), and proposed algorithm. The bases to elaborate a cervical cancer screening algorithm rely on local baseline information regarding HPV infection, and economic cost-effectiveness of the proposed algorithm. HPV vaccination programs are almost entirely supported by national health entities so that updated information is needed in order to achieve adequate and cost-effective preventive measures.

Concerning the age range of the Portuguese regional cervical cancer screening programs, all ARS implemented the starting age at 25 years (except for ARS *Lisboa e Vale do Tejo*), and all ARS designed screening programs up to 64 years (except ARS *Norte*). This is the commonest age range adopted by several European countries, as for example Finland and United Kingdom (UK), but they differ in the re-screening interval. In Finland (screening implemented since the 1960's), it is recommended a 5-year re-screening interval, as it is observed for ARS *Norte* and ARS *Lisboa e Vale do Tejo*; though in the UK (with an organized screening program since 1988), the re-screening interval is variable according to age, so that women aged 25 to 49 years are re-screened every three years, and women aged 50 to 64 are re-screened every five years [51; http://www.cancer.fi/; http://www.cancerscreening.nhs.uk/cervical/index.html]. This 3-year re-screening interval is also adopted by three Portuguese ARS. Worldwide, there are countries that present an enlarged screening age range, such as Australia which screen women aged 20 to 70 years, with a 2-year re-screening interval (screening implemented since 1991) [http://www.cancerscreening.gov.au/]. In the USA, it was also adopted a variable re-screening interval for women aged 21 to 65 years (every three years), but if preferred, the women aged 30 to 65 years that want to lengthen the re-screening interval, a co-testing approach of cytology and HPV DNA testing with a 5-year re-screening interval may be agreed [http://www.uspreventiveservicestaskforce.org/uspstf/uspscerv.htm].

All cervical cancer screening programs discussed here have a regional approach, but there are some variations about the primary screening test within the different programs. In Portugal, only ARS *Centro* proposes the Pap smear in a cytology-based algorithm, as adopted in Finland, which is

the most successful European cervical cancer screening program. In fact, in this Northern European country, cervical cancer incidence and mortality rates have decreased greatly over these last decades (four of five cancers are prevented), so that currently no HPV vaccine has been introduced in the Finnish national immunization program [52]. In the UK, cervical cancer screening started by performing Pap smears, but in 2003 changed to LBC [http://www.cancerscreening.nhs.uk], though Australia and USA still perform the Pap smear as the primary screening test [http://www.cancerscreening.gov.au/; http://www.cdc.gov/cancer/cervical/statistics].

Pap tests are notoriously insensitive to detect cervical lesions, as they only detect 50.0 to 75.0% of the cancer cases. However, repeated cytological screening episodes might compensate test's insensitivity, increasing its global performance. Nonetheless, Pap smears feasibility may be restricted to developed countries, so that HPV DNA tests can constitute an alternative for primary screening, extending the re-screening interval, due to their 90.0 to 95.0% sensitivity to detect precancerous cervical lesions. Despite this high sensitivity, HPV DNA tests only detect HPV infection, failing the identification of cell abnormalities that precede cervical cancer, which are successfully detected through cytology.

Regarding vaccination, the UK introduced HPV vaccines into the national immunization program in 2008 destined to vaccinate young girls aged 12 to 13 years, with a catch-up program for girls aged up to 18 years (finished in 2011). Despite it started with the bivalent HPV vaccine, in 2011 the UK Department of Health deliberated that HPV vaccination program should switch to the quadrivalent vaccine (from September 2012), mainly because of the increased costs of the national health system to treat the elevated number of genital warts [52; www.patient.co.uk]. Contrary to the implemented in Portugal, the HPV vaccination program in UK has a school-based approach. Also, the Australian national school-based HPV vaccination program is destined to vaccinate young girls aged 12 to 13 years, and has been extended to include young boys since February 2013 (free of charge) [http://hpv.health.gov.au/]. HPV vaccination was introduced in the USA in 2006 (quadrivalent vaccine; in 2009 the bivalent vaccine was introduced) along with a recommendation for vaccinating young girls and women aged 11 to 26 years; the quadrivalent HPV vaccine was also recommended for young boys and men aged 11 to 26 years [53].

HPV vaccines represent the ultimate cervical cancer prevention strategy, but it should take several years until a real impact on the decrease of cervical cancer incidence rates can be observed. On the other hand, cervical cancer screening must continue, as the majority of sexually active women have been exposed to HPV infection prior to the implementation of vaccination schemes, and they are at risk of developing cervical cancer [46,54].

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CHAPTER *3*

Ana Gradíssimo, Nuno Verdasca, Ângela Pista

Contributions of the Authors:

Ana Gradíssimo was responsible for the research, writing and organization of this paper. She was also responsible for all the data analysis, including figures and tables, as well as laboratory work, namely DNA extraction and HPV genotyping.

Nuno Verdasca contributed with proofreading and image rearrangements. He also conducted laboratory work, including DNA extraction and HPV genotyping.

Ângela Pista contributed with scientific guidance.

ORIGINAL ARTICLE

Prevalence of Human Papillomavirus in a Group of Women from Opportunistic Screening

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Running Title: HPV Prevalence in Opportunistic Screening in Portugal

ABSTRACT

Persistent high-risk human papillomavirus (HPV) infection is the major risk factor for cervical cancer development. Portugal has one of the highest incidence rates for cervical cancer in Europe. The objective was to assess the overall and age-stratified HPV prevalence in an opportunistic screening setting to further improve screening policies and management of HPV-infected Portuguese women. A total of 2149 samples were selected from different regions in Portugal. Samples were stratified by age and cytological groups. HPV DNA detection and genotyping was performed using CLART HPV 2 assay. The overall HPV prevalence was 42.2% (906/2149; 95% CI: 40.1-44.2). After age stratification of cases, the proportion of HPV infection was higher for the age group 25 to 29 years, and then decreasing with increasing age. The most frequently detected genotypes were HPV 16, 31, 53, 51, and 66, and there was a statistically significant association between the prevalence of infection with HR-HPV genotypes and age ($P = 0.005$). HPV DNA testing revealed that 22.6% (273/1209) of the samples with normal cytology were HPV-positive; moreover, HPV prevalence increased significantly with the severity of lesions ($P < 0.001$). HPV infection was frequent, since the present evaluation relies on opportunistic screening of women who presented clinical diagnosis suggestive of HPV infection (potential increase and overstated HPV prevalence). Also, the age-specific distribution of HPV infection might be useful for detection strategies, by determining how and when to act for preventing cervical cancer development.

Keywords: Human papillomavirus, genotyping, HPV genotype-specific distribution, cervical cancer.

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INTRODUCTION

Human papillomavirus (HPV) is a common sexually transmitted infection (STI) and is responsible for a wide range of anogenital diseases that represent a high burden of public health disease in both women and men. Approximately 80.0% of sexually active adults will be infected with HPV during their lifetime. Worldwide, prevalence of HPV infection is estimated to be from 9.0 to 13.0%, and in Europe it varies from 3.0 to 15.0%. Most infections are asymptomatic and clear spontaneously, but persistent infections can progress to precancerous lesions and cancer [1]. The critical factor influencing the likelihood of progression to cervical cancer is the infecting HPV genotype. To date, more than 100 HPV genotypes have been identified [2]. According to their oncogenic potential, HPV genotypes can be subdivided into low-risk (LR-HPV) genotypes, v.g. HPV 6 and 11 (mainly found in genital warts), and high-risk (HR-HPV) genotypes, v.g. HPV 16 and 18, that are frequently associated with low- or high-grade cervical abnormalities (putative precursors for cervical cancer), and invasive cervical cancer [3].

Persistent infection with HR-HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) is considered a strong predictor of development of high-grade cervical lesions and cervical cancer [4,5]. HPV DNA detection tests have proven to be more sensitive than cytology, by revealing the presence of multiple HPV infections in women with and without cytological abnormalities. To date, 20.0 to 50.0% of HPV-positive women evidenced infection with multiple HPV genotypes [6-8]. Co-infection with more than one HPV genotype has been observed more frequently among young women and among those with cytological abnormalities [9-13]. However, the clinical importance of these multiple HPV infections in cervical cancer development remains unclear. Several studies have evidenced that multiple HPV infections seem to be associated with a significantly increased risk of high-grade lesions as compared with single infections [10,14,15].

Other risk factors that promote carcinogenesis are either biological, such as co-infections with other STIs, or behavioral, such as sexual habits. A consistent association has been demonstrated between an increased lifetime number of sexual partners and HPV infection among women [16-18]. However, different factors, such as young age at first sexual intercourse, use of oral contraceptives, and smoking have shown inconsistent associations with HPV infection [17,19-21].

In Portugal, cervical cancer is the fourth most frequent cancer in women, and the second most frequent cancer in women aged 15 to 44 years of age. The age-standardized incidence rate is 12.2 per 100,000 women, with 949 new cases of cervical cancer diagnosed per year, and an age-standardized mortality rate of 3.6 per 100,000 women, with a total of 346 deaths as a consequence of cervical cancer [22].

The objective of the present study is to assess the overall and age-stratified prevalence of HPV, in normal and abnormal cytological smears, as well as to determine genotype-specific

proportion of HR-HPV genotypes. This evaluation is intended to gain knowledge and information about HPV infection among Portuguese women, in order to further improve screening policies.

MATERIAL AND METHODS

Study Design

A total of 2149 samples were selected for the study from different regions of Portugal, during 2009 to 2011. The selection criteria included (1) women aged 18 to 65 years; (2) women sexually active; and (3) referral from a gynecology clinic. Criteria for exclusion were (1) pregnancy; (2) history of atypical cytology, cervical intraepithelial neoplasia (CIN) or treatment for cervical disease in the prior 12 months; and (3) having had a hysterectomy. All samples were stratified by age group: 18-19; 20-24; 25-29; 30-39; 40-49; 50-59; 60-65 years. Eligible women attended to the Human Papillomavirus Reference Laboratory at the National Institute of Health (INSA) for opportunistic screening and HPV testing, as they evidenced a clinical diagnosis suggestive of HPV infection. The study group included a high proportion of women at risk for cervical cancer with a broad range of outcomes and an increased rate of disease cases. Cervical cell samples were collected with a cytobrush during clinical examination for cytology (Pap smears). Liquid-based cytology (LBC) samples were also collected for further HPV testing. Pap smears were classified as normal smears (NILM), atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells that cannot exclude high-grade lesions (ASC-H), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and invasive cervical cancer (ICC).

Laboratory Methods

HPV DNA was isolated from 1 ml of cellular suspension using the automated extraction system NucliSENS® easyMAG® (bioMérieux, Boxtel, Netherlands), as specified in the manufacturer's instructions. Nucleic acids were eluted in a final volume of 100 µl and stored at -20ºC until use for PCR analysis.

Detection and genotyping of HPV were carried out using the commercial system CLART® Papillomavirus Humano 2 (CLART) assay (Genomica, Madrid, Spain) according to the manufacturer's instructions. This methodology uses biotinylated primers that amplify a 450 bp fragment within the L1 region, which allows the identification of 35 genotypes (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89). Co-amplification of an 892 bp region of the human housekeeping gene CFTR (*cystic fibrosis transmembrane conductance regulator*) and a 1,202 bp fragment of a transformed plasmid, provide a control to ensure DNA extraction adequacy and PCR efficiency, avoiding false-negative results. HPV genotyping was performed by hybridization in a low-density microarray

containing triplicate DNA probes specific to each one of the 35 genotypes included in the assay. Semi-quantitative results were obtained in an automatic reader. The clinical sensitivity and specificity of CLART assay has been previously established as 96.9% and 71.9%, respectively (Chapter 4) [23].

Statistical Methods

For statistical purposes, HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82 were considered to be HR-HPV (including both probable and possible HR genotypes), and HPV 6, 11, 40, 42, 43, 44, 54, 61, 62, 71, 72, 81, 83, 84, 85, and 89 were considered to be LR-HPV (including undetermined genotypes) according to the classification proposed elsewhere [24,25].

HPV genotype-specific proportion according to cervical lesions distribution was investigated, as well as risk behavior variables for HPV acquisition (age, number of lifetime partners, and use of contraceptives). Data was described including mean, standard deviation (SD), median, range and both absolute and relative frequencies. The overall and age-stratified prevalence of HPV infection and genotype-specific distribution of HPV are presented as relative frequencies with two-sided 95% confidence intervals (CI). Bivariate analysis (Pearson χ^2 test) was used to (i) evaluate the relationship between HPV prevalence and age group or region, (ii) compare the proportion of HPV genotype according to the cytological result, and (iii) compare single versus multiple HPV infections.

To determine the proportion of HR-HPV and LR-HPV genotypes, cases were counted more than once whenever they harbored a multiple infection, comprehending a mixture of HR and LR genotypes. The proportion of individual HPV genotypes was determined as they were identified either in single or in multiple infections. Multiple HPV infections were compared with single HPV infections in order to assess the risk for cervical cancer development. The odds ratio (ORs), together with two-sided 95% CI, were done using 2 x 2 contingency tables, and the corresponding P value was calculated using the chi-square test or, when appropriate, Fisher's exact test. Statistical analyses were performed using SPSS software version 20.0 (IBM Corporation Inc., Armonk, NY, USA).

RESULTS

Study Samples

Almost half of the studied samples (48.9%; 1051/2149) were from Lisbon area (Fig.1). Two other large groups of samples were recruited from the north of mainland Portugal (28.2%; 605/2149) and from the Azores islands (19.0%; 409/2149); two smaller groups were recruited in the south (3.2%; 68/2149) and centre of mainland Portugal (0.7%; 16/2149). Characteristics of the 2149 samples included in the study are summarized in Table 1. The mean age was 35 years, 4 months (SD: 10 years; median age 33 years). The majority of women had no prior history of any STI, about half

reported having more than one sexual partner during their lifetime, and the use of oral contraceptives was more frequent than the use of condom.

Fig. 1: Geographical distribution of the selected cases.

Table 1: Characteristics of study samples

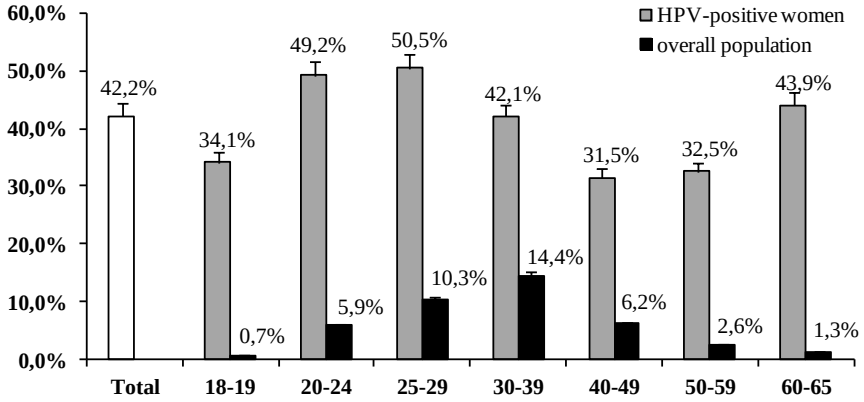
Characteristic	Study Sample (n=2149)
Age, mean (SD)	35.4 (10.5)
Age distribution in years, n (%)	
18-19	44 (2.0)
20-24	256 (11.9)
25-29	438 (20.4)
30-39	737 (34.3)
40-49	422 (19.6)
50-59	169 (7.9)
60-65	66 (3.1)
Data missing*	17 (0.8)
Prior history of STI, n (%)	
No	1841 (85.7)
Yes	291 (13.5)
Data missing*	17 (0.8)
Lifetime number of sexual partners, n (%)	
1	29 (1.3)
2	13 (0.6)
3-5	25 (1.2)
>=6	2 (0.1)
Data missing*	2080 (96.8)
Contraceptive use, n (%)	
Yes	104 (4.8)
Oral contraceptive	80 (3.7)
Condom	10 (0.5)
Other method	14 (0.6)
No	5 (0.2)
Data missing*	2040 (94.9)

*Not determined, unknown.

HPV Prevalence

The overall HPV prevalence was 42.2% (906/2149; 95% CI: 40.1-44.2) (Fig.2). After age stratification of cases, the proportion of HPV infection was higher for age groups 20 to 24 years (49.2%, 126/256; 95% CI: 47.1-51.3) and 25 to 29 years (50.5%, 221/438; 95% CI: 48.4-52.6), and then decreasing with increasing age. The calculated OR for HPV infection was 1.237 (95% CI:0.693-2.209; P = 0.491) for women with 20 to 24 years, and was 1.299 (95% CI: 0.748-2.259; P = 0.357)

for women aged 25 to 29 years. The calculated HPV prevalence in the study samples by age group is presented in Fig.2, where a second peak was observed in women aged 60 to 65 years (43.9%, 29/66; 95% CI: 41.8-46.0). There was a statistically significant association between HPV prevalence and age (P < 0.001).



Age Group, years	No. Women Tested	No. Women HPV-positive	95% CI	OR (95% CI)
Total	2149	906	40.1 – 44.2	
18-19	44	15	32.1 – 36.1	0.660 (0.277 – 1.563)
20-24	256	126	47.1 – 51.3	1.237 (0.693 – 2.209)
25-29	438	221	48.4 – 52.6	1.299 (0.748 – 2.259)
30-39	737	310	40.0 – 44.2	0.926 (0.542 – 1.586)
40-49	422	133	29.5 – 33.4	0.587 (0.336 – 1.029)
50-59	169	55	30.5 – 34.4	0.616 (0.330 – 1.149)
60-65	66	29	41.8 – 46.0	1.00 (reference)
P*			<0.001	

*Pearson χ^2 test.

Fig. 2: Human papillomavirus prevalence stratification by age group (years), with standard error bars.

Genotype-Specific HPV Prevalence

Among HPV-positive samples, HR-HPV genotypes were identified in 96% (869/906) of the cases. In total, 34 different genotypes (19 HR and 15 LR genotypes) were detected. The most common genotypes were HPV 16 (17.5%; 95% CI: 15.9-19.1), HPV 31 (8.1%; 95% CI: 6.9-9.3), HPV 53 (4.3%; 95% CI: 3.4-5.2), HPV 51 (4.2%; 95% CI: 3.3-5.1), HPV 18 (3.9%; 95% CI: 3.1-4.7), and HPV 66 (3.6%; 95% CI: 2.8-4.4) (Table 3). The proportion of HPV 16 among positive cases was 41.3% (374/906), while the proportion of HPV 18 among positive cases was only 9.4% (85/906). The calculated OR for the development of a high-grade cervical lesion associated to HPV 16 and/or 18 infection was 2.785 (95% CI: 2.020-3.844; P < 0.001), when compared to other HR-HPV genotypes.

HPV genotypes included in bivalent (HPV 16 or 18) and quadrivalent (HPV 6, 11, 16, or 18) HPV vaccines were detected in 50.3% (456/906) and 54.0% (489/906) of the HPV-positive cases, respectively, and in 21.2% (456/2149; 95% CI: 19.5-22.9) and 22.7% (489/2149; 95% CI: 20.9-24.5) of all the study samples, respectively. Among HPV-positive samples, one HPV genotype included in the quadrivalent vaccine was detected in 54.0% (489/906) of the cases, while two HPV genotypes

included in the quadrivalent vaccine were detected in 3.0% (27/906) of the cases and only 0.3% (3/906) of the cases revealed to be infected by three of the HPV genotypes included in the quadrivalent vaccine. There were no cases involving the simultaneous detection of the four vaccine HPV genotypes.

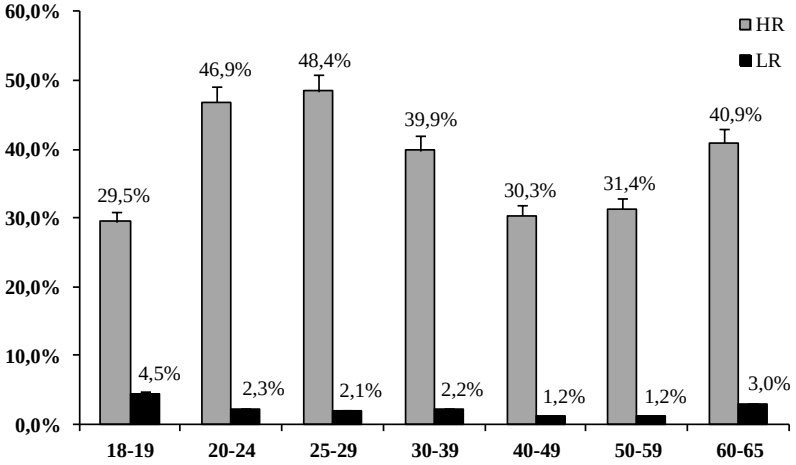
Table 3: Distribution of HPV genotypes in positive cases, and prevalence of HPV genotypes in the study samples

HPV Genotype	No. Times That Each HPV Was Detected (S + M)	% of Genotype-Specific HPV in Infected Samples (n = 906)†	% of Genotype-Specific HPV (n = 1600)*	Prevalence (%) in Study Samples (n = 2149)
High-Risk				
16	**209 + 168**	**41.6**	**23.6**	**17.5**
18	**41 + 44**	**9.4**	**5.3**	**3.9**
26	0 + 2	0.2	0.1	0.01
31	79 + 95	19.2	10.9	8.1
33	18 + 47	7.2	4.1	3.0
35	7 + 23	3.3	1.9	1.4
39	2 + 21	2.5	1.4	1.1
45	10 + 28	4.2	2.4	1.7
51	23 + 68	10.0	5.7	4.2
52	19 + 52	7.8	4.4	3.3
53	22 + 70	10.2	5.7	4.3
56	15 + 26	4.5	2.6	1.9
58	12 + 34	5.1	2.9	2.1
59	6 + 40	5.1	2.9	2.1
66	18 + 58	8.3	4.7	3.6
68	2 + 24	2.8	1.6	1.2
70	2 + 25	3.0	1.7	1.3
73	3 + 15	2.0	1.1	0.8
82	2 + 19	2.3	1.3	1.0
Low-Risk				
6	**3 + 22**	**2.8**	**1.6**	**1.2**
11	**3 + 5**	**0.9**	**0.5**	**0.4**
40	1 + 7	0.9	0.5	0.4
42	15 + 27	4.6	2.6	1.9
44	2 + 6	0.9	0.5	0.4
54	5 + 17	2.4	1.4	1.0
61	5 + 34	4.3	2.4	1.8
62	5 + 26	3.4	1.9	1.4
71	0 + 2	0.2	0.1	0.08
72	1 + 1	0.2	0.1	0.08
81	5 + 17	2.1	1.4	0.9
83	1 + 7	0.9	0.5	0.4
84	2 + 27	3.2	1.8	1.3
85	1 + 1	0.2	0.1	0.08
89	1 + 2	0.3	0.2	0.1

S, single infections; M, multiple infections; †The sum of percentages is higher than 100% because samples could be infected with more than one HPV genotype; *Number of times each genotype was identified either on single or multiple infections. HPV genotypes included in the quadrivalent vaccine are highlighted in bold. Most common genotypes are underlined.

There was a statistically significant association between the prevalence of infection with HR-HPV genotypes and age (P = 0.005). Stratification of the results by risk of HPV genotype (HR and

LR) and age group, showed that infections with HR-HPV genotypes were more frequent among younger age groups (20-24, 25-29, and 30-39 years) (Fig.3). The prevalence of HR-HPV infections were highest in women aged 25 to 29 years (48.4%; 95% CI: 43.7-53.0) and LR-HPV infections were highest in women aged 18 to 19 years (4.5%; 95% CI: 0.0-10.6), respectively. The prevalence of LR-HPV genotypes decreased with increasing age.



Age Group, Years	No. Samples Tested	HPV Risk Genotype			
		High-Risk		Low-Risk	
		No. Samples Infected*	95% CI	No. Samples Infected*	95% CI
Total	2149				
18-19	44	13	16.0 – 43.0	2	0.0 – 10.6
20-24	256	120	40.8 – 53.0	6	0.4 – 4.2
25-29	438	212	43.7 – 53.0	9	0.7 – 3.4
30-39	737	294	36.3 – 43.4	16	1.1 – 3.2
40-49	422	128	25.9 – 34.7	5	0.1 – 2.2
50-59	169	53	24.4 – 38.3	2	0.0 – 2.8
60-65	66	27	29.0 – 52.7	2	0.0 – 7.1
P†			0.005		

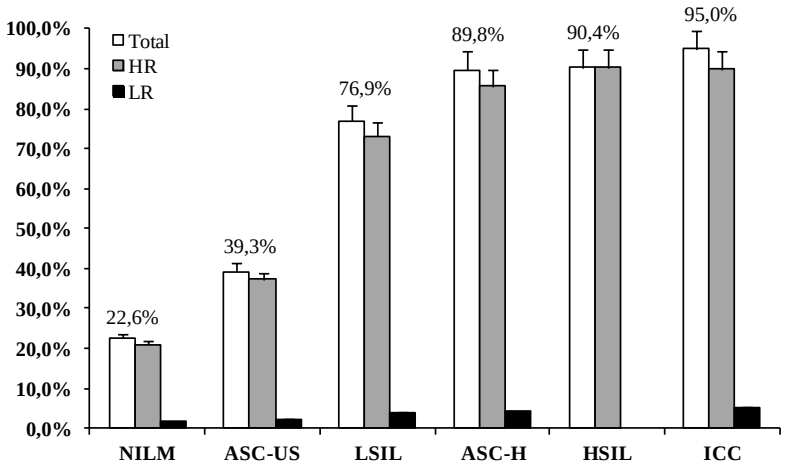
High-risk (HR) group included HPV genotypes of probable high-risk. Low-risk (LR) group included HPV genotypes of undetermined risk. CI, confidence interval. *Samples could be infected with HR-HPV and LR-HPV genotypes. †Pearson χ^2 test.

Fig. 3: Prevalence stratification in high-risk and low-risk HPV infection by age group (years), with standard error bars.

HPV Prevalence Related to Cytology

Normal cytology was observed in 56.3% (1209/2149) of the cases, 15.6% (336/2149) were ASC-US, 15.7% (338/2149) were LSIL, 2.3% (49/2149) were ASC-H, 9.2% (197/2149) were HSIL, and 0.9% (20/2149) was ICC. HPV DNA testing revealed that 22.6% (273/1209) of the samples with normal cytology were HPV-positive (Fig.4); moreover, HPV prevalence increased significantly with the severity of lesions ($P < 0.001$), namely among LSIL (76.9%; 260/338), ASC-H (89.8%; 44/49), HSIL (90.4%; 178/197), and ICC (95.0%; 19/20).

HR-HPV genotypes were identified in normal cytology cases (20.9%, 253/1209; 95% CI: 18.6-23.2), while only 1.7% (20/1209; 95% CI: 0.9-2.3) were infected by LR-HPV genotypes. In fact, HR-HPV genotypes were highly frequent, compared to LR-HPV genotypes, independently of the cytological diagnosis, being responsible for almost all cases of HPV infection (Fig.4). Consequently, there was a statistically significant association between infection with HR-HPV genotypes and cytological abnormalities (P = 0.007).



Cytology Result	Prevalence, % (95% CI) (n = 2149)	
	High-Risk	Low-Risk
NILM	20.9 (18.6 – 23.2)	1.7 (0.9 – 2.3)
ASC-US	37.2 (34.1 – 44.5)	2.1 (0.5 – 3.6)
LSIL	73.1 (68.4 – 77.8)	3.8 (1.8 – 5.8)
ASC-H	85.7 (75.9 – 95.5)	4.1 (1.4 – 9.6)
HSIL	90.4 (86.2 – 94.5)	0.0
ICC	90.0 (76.9 – 100.0)	5.0 (0.0 – 14.5)
P*	0.007	

High-risk (HR) group included HPV genotypes of probable high-risk. Low-risk (LR) group included HPV genotypes of undetermined risk. NILM, normal; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells that cannot exclude HSIL; HSIL, high-grade squamous intraepithelial lesion; ICC, invasive cervical cancer. CI, confidence interval. *Pearson χ^2 test.

Fig. 4: Prevalence stratification in high-risk and low-risk HPV infection by cytological diagnosis, with standard error bars.

When the detection of HPV 16 or 18 was evaluated regarding age and high-grade cervical lesions or cervical cancer cases, it was observed that HPV 16 was identified in normal cytologies among women with a mean age of 31.8 years (SD: 9.7 years), while for high-grade lesions the mean age was 34.7 years (SD: 9.6 years), and for cervical cancer cases was 47.3 years (SD: 16.5 years). For HPV 18 it was 32.2 years (SD: 9.2 years), 34.0 years (SD: 7.5 years), and 43.3 years (SD: 13.4 years), respectively, suggesting a faster progression to cervical cancer when HPV 18 is the infecting genotype. Moreover, HPV 16 and 18 are highly associated with the progression from cervical lesions to cervical cancer (Fig.5).

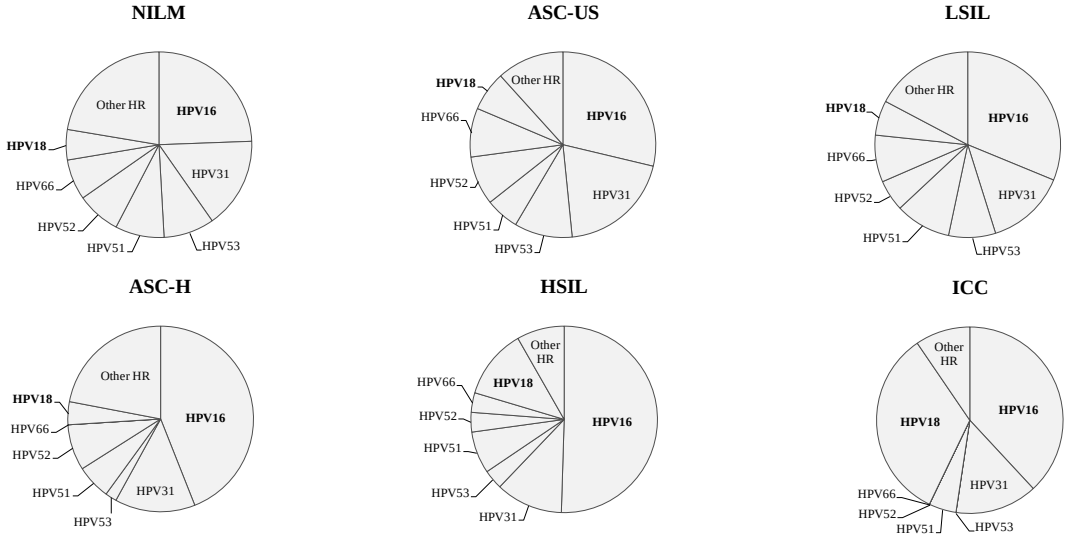
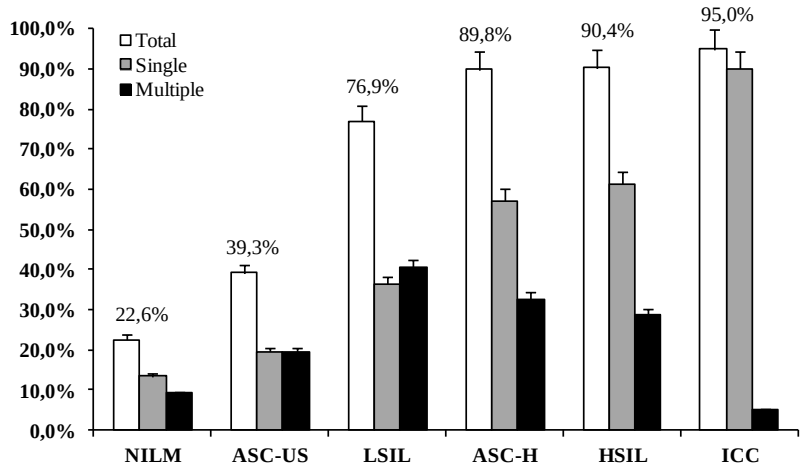


Fig. 5: Contribution of the most frequent HPV genotypes by cytological diagnosis.

Multiple HPV Infections

Infection with multiple HPV genotypes was identified in 18.1% (388/2149; 95% CI: 16.4-19.7) of the study samples, representing 42.8% (388/906) of the total HPV-positive cases. While 57.2% (518/906) of the infections were caused by a single HPV genotype, 217 (23.9%) encompassed two HPV genotypes, 113 (12.5%) three genotypes, and 58 (6.4%) at least four genotypes. The most common co-infection was exclusively by HR-HPV genotypes (23.4%; 212/906) (HPV 16+31, 3.1%) whereas infections comprehending HR-LR and LR-LR HPV genotypes were identified in 18.9% (171/906) (HPV 6+16, 1.1%) and 1.0% (9/906) (HPV 61+62, 0.2%), respectively, (representing 9.9%, 8.0%, 0.4% among the overall studied samples). The prevalence of multiple infections was higher among women under 30 years (Fig.6). The lowest prevalence of multiple infections was observed in women aged 50 to 59 years (7.1%; 12/169). Consequently, a significant association between the presence of multiple infections and young age ($P < 0.001$) could be established.

According to cytological diagnosis, multiple HPV infections were less frequent in HPV-positive cases of normal cytology (9.2%; 111/1209) and ICC (5%; 1/20) (Fig.6). The proportion of multiple HPV infections was higher in LSIL cases (40.5%; 137/338). Single HPV infections increased with increasing severity of cervical lesions ($P < 0.001$); the same association could be established for HR-HR HPV infections ($P < 0.001$). Women infected with multiple HPV genotypes were not at higher risk for high-grade cervical lesions development than the infected by a single HPV genotype (OR 0.495; 95% CI: 0.358-0.685) ($P < 0.001$).



Category	No. Samples HPV-Positive	No. Samples with Single HPV Genotype	Prevalence of Single HPV Genotype % (95% CI)	No. Samples with Multiple HPV Genotypes	Prevalence of Multiple HPV Genotypes % (95% CI)	P*
Total	906	518	24.1 (22.2 – 25.9)	388	18.1 (16.4 – 19.7)	0.605
18-19†	15	7	15.9 (3.0 – 28.7)	8	18.2 (6.7 – 29.5)	<0.001
20-24	126	56	21.9 (16.8 – 26.9)	70	27.3 (21.9 – 32.8)	
25-29	221	102	23.3 (19.3 – 27.2)	119	27.2 (23.0 – 31.3)	
30-39	310	184	25.0 (21.8 – 28.1)	126	17.1 (14.4 – 19.8)	
40-49	133	92	21.8 (17.9 – 25.7)	41	9.7 (6.9 – 12.5)	
50-59	55	43	25.4 (18.9 – 32.0)	12	7.1 (3.2 – 10.9)	
60-65	29	18	27.3 (16.5 – 38.0)	11	16.7 (7.7 – 25.6)	
NILM	273	162	13.4 (11.5 – 15.3)	111	9.2 (7.5 – 10.8)	<0.001
ASC-US	132	66	19.6 (15.4 – 23.8)	66	19.6 (15.4 – 23.8)	
LSIL	260	123	36.4 (31.2 – 41.5)	137	40.5 (35.3 – 45.8)	
ASC-H	44	28	57.1 (43.3 – 71.0)	16	32.7 (19.5 – 45.8)	
HSIL	178	121	61.4 (54.6 – 68.2)	57	28.9 (22.6 – 35.3)	
ICC	19	18	90.0 (76.9 – 100.0)	1	5.0 (0.0 – 14.6)	

NILM, normal; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells that cannot exclude HSIL; HSIL, high-grade squamous intraepithelial lesion; ICC, invasive cervical cancer. †Age group is represented in years. *Pearson χ^2 test.

Fig. 6: Prevalence stratification in single and multiple HPV infections by age and cytological group, with standard error bars.

DISCUSSION

HPV infection was very common among the population under evaluation, with an overall prevalence of 42.2%. Normal cytology cases evidenced HPV infection more frequently (22.6%) than the described in an enlarged study held in Portugal (16.5%) [26]. This higher proportion is explained by the different characteristics of both studies; in fact, while the present evaluation relies on opportunistic screening (potential increase and overstated HPV prevalence), the epidemiological evaluation [26] involved a large screening-type sample of Portuguese women. However, epidemiologic studies comprehending asymptomatic women of several countries, reported HPV infection estimates ranging 2.0% to 44.0% [27], which might reflect methodological differences or constitute real different HPV distribution.

As in other studies, HPV prevalence was higher in women under 30 years [28]. The peak of HPV prevalence is usually observed three years after the onset of sexual activity. The present study involved women attending clinics for opportunistic screening that were referred to INSA for HPV genotyping, following clinical diagnosis suggestive of HPV infection. This fact may help to understand that the peak of HPV prevalence was observed in women a little older than the expected (25-29 years), when compared to other studies (peak occurring at 20-24 years), considering that this population included a high proportion of women with HPV-related disease [14,20,27]. A second peak of HPV prevalence in older women is observed across some world regions, which could be due to three main aspects: an impaired immune response caused by hormonal changes at menopause, inducing reactivation of latent HPV infections; differences in sexual behaviors; or a cohort effect [29,30]. In this study, an increase in women aged 60 to 65 years could be observed, which might be related to the relatively small number of cases that were enrolled; moreover, HPV DNA testing in women over 60 years is less frequent, being only performed when cervical disease is colposcopically observed.

The most common HPV genotypes were HPV 16, followed by HPV 31, 53, 51, 18, and 66, with prevalence rates ranging from 17.5% to 3.6%, which is mainly in accordance with other Portuguese published data [31]. In a comparison study comprehending single and multiple infections, HPV 66 and 58 were found among the most prevalent genotypes, while HPV 18 was poorly detected [31]. In the present study, the higher disease rate, along with the high number of older women, may affect HPV genotype distribution, especially for HPV 18 which is more associated with an aggressive behavior and a rapid progression for cervical cancer [32]. In fact, the mean age at diagnosis of high-grade lesions associated to HPV 16 or 18 infections was 34.7 years and 34.0 years, respectively, while for cervical cancer was 47.3 years and 43.3 years, respectively. In the present study, HPV 16 was the most frequent genotype (51.6%; 112/217) followed by HPV 18 (14.7%; 32/217) among high-grade lesions and ICC cases. The prevalence of these two genotypes increased with the severity of cervical neoplasia, which is in line with previous reports [33-35]. These two oncogenic genotypes are responsible for more than 65.0% of high-grade cervical lesions, so HPV vaccines may become responsible for a high decrease on HPV 16/18-associated diseases in Portugal [26]. HPV 16 was the most commonly detected genotype independently of cytological diagnosis and age, while HPV 18 was more frequent among women older than 30 years and it was mainly identified in precancerous lesions and cervical cancer, suggesting a higher risk for progression linked to HPV 18 infection as recently suggested by others [35,36].

The high prevalence of HPV 31, 53, 51 and 66 found has been described by other authors [12,37,38]. Recent migrations from regions where incidence rates for those HPV genotypes are high, such as Brazil and sub-Saharan Africa, might explain the circulating proportions of these genotypes among Portuguese women, and support the need for continuously evaluate genotype-specific HPV distributions, as putatively rare HR-HPV genotypes might become frequent, and health measures may

have to be implemented in accordance to those molecular epidemiological changes. However, HPV 31 seems to be widespread across Europe and HPV 51 and 53 have been consistently reported in several Southern European studies [38-41].

Multiple HPV infections were detected in 18.1% of the studied samples, representing 42.8% of the HPV-positive cases. The proportion of multiple genotypes was higher than the previously reported for a similar population [31]. These infections were detected more frequently in young women, who could have higher number of sexual partners. In fact, young age is more often associated with an increased rate of multiple HPV infections, suggesting a demographic and behavioral influence in HPV acquisition, mostly probably through multiple sexual contacts [8,10,13,33]. According to cytological diagnosis, multiple HPV infections were more frequent in abnormal smears and low-grade cervical lesions, and decreased among high-grade cervical lesions, possibly indicating that co-infection with multiple genotypes are common and occur independently of the severity of cytological diagnosis. The presence of multiple HPV infections was not significantly associated with the development of high-grade cervical lesions, suggesting that might not constitute a risk factor for carcinogenesis [11,42,43]. The data reported here contradicts what was reported in another Portuguese study, where multiple HPV infections were associated with persistency and seemed to represent a higher risk for the development of precancerous lesions [31]. In fact, more important than the influence of the presence of multiple HPV genotypes, might be the individual oncogenic potential of each infecting genotype, as in this study HR-HPV infection represented an increased risk for cervical cancer development.

This study highlights the importance of HPV genotype evaluation in opportunistic screening and furnishes baseline data that will contribute for the optimization of routine screening policies for Portuguese women. Also, the definition of age-specific distribution for HPV infection might help HPV detection strategies, by determining how and when to act for preventing cervical cancer development.

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CHAPTER 4

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Contributions of the Authors:

Ângela Pista was responsible for the research, writing and organization of this paper. She was also responsible for laboratory work, namely Hybrid Capture® 2 High-Risk HPV DNA Test (HC2) tests.

Nuno Verdasca was responsible for the majority of laboratory work, including DNA extraction and HPV genotyping. He also contributed with proofreading.

Ana Oliveira performed the majority of HC2 tests, and contributed with proofreading and the extensive data analysis and image arrangements.

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Clinical Performance of the CLART Human Papillomavirus 2 Assay Compared With the Hybrid Capture 2 Test

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Persistent infection by high-risk human papillomavirus (HR-HPV) is a cause of cervical cancer. The use of HPV detection in cervical screening programs may improve the ability to identify women at risk of cervical cancer. Therefore, the development of appropriate methods for the detection of HR-HPV is essential. The aim of this study was to evaluate the clinical performance of the CLART Human Papillomavirus 2 assay (CLART) in comparison with the Hybrid Capture 2 test (HC2), using a clinical cut-off of cervical intraepithelial neoplasia grade 2 or worse. Discrepant results were analyzed further by the PapilloCheck HPV genotyping system. In the 425 studied women, HR-HPV positivity rates were similar by both tests (CLART-13 HR-HPV: 63.1%; CLART-17 HR-HPV: 64.7%; HC2: 64.5%). Agreement between CLART-13 HR-HPV ($\kappa = 0.969$; concordance level 98.6%), CLART-17 HR-HPV ($\kappa = 0.974$; concordance level 98.8%), and HC2 were very good. When 13 HR-HPV types were considered, the two tests showed a clinical sensitivity of 96% (95% CI: 92.6–97.9). The clinical specificity of CLART-13 HR-HPV was 73.6% (95% CI: 66.7–79.5) for cervical intraepithelial neoplasia grade 2 or worse, which was comparable to HC2 (71.4%; 95% CI: 64.3–77.5). When all 17 HR-HPV types were considered, CLART showed a clinical sensitivity of 96.9% (95% CI: 93.8–98.5) and a clinical specificity of 71.9% (95% CI: 64.9–78.0). In conclusion, the CLART assay is efficient, sensitive, reproducible, and has a similar performance to HC2 for cervical intraepithelial neoplasia grade 2 or worse. Furthermore, this assay has the advantage of detecting and genotyping 35 HPV types by a single test, which can provide additional information on the predictive value of infection with HR-HPV. ***J. Med. Virol. 83:272–276, 2011.*** © 2010 Wiley-Liss, Inc.

KEY WORDS: HPV; CLART Human Papillomavirus 2; HC2; screening; genotyping

INTRODUCTION

Epidemiological studies have established that certain human papillomavirus (HPV) genotypes are related etiologically to cervical cancer development [Koutsky et al., 1992; Schiffman et al., 1993; Bosch et al., 1995]. More than 120 HPV types have been identified, of which approximately 40 can infect the mucosa of the genital tract. According to the oncogenic potential, these HPV types can be classified into high-risk (HR), associated with premalignant lesions and cervical cancer, and low-risk (LR), found mainly in benign lesions [Munoz et al., 2003; Bernard et al., 2010]. Most infections will clear spontaneously, but persistent infection with HR-HPV is a strong predictor of the development of high-grade cervical intraepithelial neoplasia (CIN) and cancer of the cervix uteri [Nobbenhuis et al., 1999; Kjaer et al., 2002].

In areas where the Pap smear is the primary screening method, the development of premalignant lesions may be attributed to the low reproducibility and sensitivity of the Papanicolau test [Renshaw, 2002]. Several studies [Bosch and de Sanjose, 2003; Cuzick et al., 2003, 2008; Bulkmans et al., 2007; Szarewski et al., 2008] have shown that the combined use of cytology and HPV DNA testing in women above 30 years old can improve the sensitivity and the negative predictive value (NPV) of screening. HPV testing can also provide the reassurance of extended intervals

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between screenings, and can be cost-effective for the detection of high-grade lesions in women with equivocal cytological abnormalities. Indeed, recent studies [Castle et al., 2005; Lai et al., 2007] have shown that a single positive result for either HPV 16 or 18 has a high predictive value for CIN grade 2 or worse (≥CIN2). Several HPV assays with various levels of sensitivity and specificity have been made available recently. However, clinical validation is required before these tests can be used as a "stand-alone" method in cervical cancer screening programs. Tests with significantly higher sensitivity for the detection of HPV DNA than the Hybrid Capture 2 test (HC2; Qiagen, Hilden, Germany) could detect latent infections that are irrelevant clinically, which may lead to overtreatment of women [Snijders et al., 2003; Meijer et al., 2009]. Recently, an assay based on PCR and hybridization, the CLART Human Papillomavirus 2 (CLART) (Genomica, Madrid, Spain) was developed for the detection and genotyping of 35 HPV types (20 HR-HPV and 15 LR-HPV), in single or multiple infections.

The aim of this study was to evaluate the performance of the CLART assay in comparison with the HC2 test in women for whom histological results were available. Further, the clinical performance was evaluated for each histological grade, using a clinical cut-off of CIN grade 2 or worse (≥CIN2).

MATERIALS AND METHODS

Study Design

The study population comprised 425 archived cervical samples from sexually active women, attending at primary Health-Care Clinics of the National Health Service and Gynaecological Outpatient Clinics. Although not a screening population, the advantage was a broad range of outcomes and a high disease rate, which would enable accurate evaluation of sensitivity and specificity in a relative small sample. All cervical samples were collected in ThinPrep PreservCyt medium (Cytyc UK, Crawley, West Sussex, UK) during clinical examination for cytological analyses. The residual liquid-based cytology (LBC) was used for HPV testing. In 405 samples, the final clinical diagnosis was based on histological examination of biopsy samples obtained at colposcopy. As suggested by Wentzensen et al. [2009] and according to histology, 178 out of the 405 women (44%) were considered to have CIN grade 1 or less (≤CIN1, regarded as controls), while 227 (56%) were diagnosed as CIN grade 2 or worse (≥CIN2, regarded as cases). No patient was sampled solely for the purpose of this research study.

All samples were tested by both the CLART and the HC2 assays. Discrepant results were analyzed further using the PapilloCheck HPV genotyping system (Greiner Bio-One, Frickenhausen, Germany). To assess the reproducibility of the CLART assay, 75 samples were retested randomly twice by two operators.

Each HPV test was carried out independently of each other according to the manufacturer's instructions. DNA was isolated from 1 ml of cellular suspension by using the NucliSENS easyMAG (BioMerieux, Boxtel, The Netherlands) system, as specified in the manufacturer's instructions. Nucleic acids were eluted in a final volume of 100 µl and stored at −20°C until use for PCR analysis.

CLART Human Papillomavirus 2

This methodology uses biotinylated primers that amplify a fragment a 450 bp fragment within the HPV L1 region. Co-amplification of an 892 bp region of the CFTR gene and a 1,202 bp fragment of a transformed plasmid provides a control to ensure DNA extraction adequacy and PCR efficiency. Amplicons are detected by hybridization in a low-density microarray containing triplicate DNA probes specific to 35 types (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89). Semi-quantitative results can be obtained in an automatic reader.

Hybrid Capture 2

The HC2 test is a sandwich capture molecular hybridization assay that uses a signal amplification detection method based on chemiluminescence. Thirteen HR-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) can be detected with this test. The resultant DNA:RNA hybrids are captured on a microplate, and the emitted light is measured in a luminometer as relative light units (RLU). Samples were considered as positive if the ratio RLU/cut-off was >1.0 (equivalent to 1.0 pg HPV DNA/ml). All the cut-offs between 1 and 2.5 were retested and all were >1.0 RLU/cut-off (data not shown).

PapilloCheck HPV Genotyping

The PapilloCheck system allows the genotyping of 24 HPV types (HPV 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 43, 44/55, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82). This assay uses a multiplex PCR that amplify a 350 bp fragment of the E1 gene of HPV. Co-amplification of the human gene ADAT1 is used as an internal control. The hybridization is performed on a microarray chip, which is automatically scanned and analyzed using the CheckScanner™ at both 532 and 635 nm and the CheckReport software, respectively.

Statistical Analysis

For comparative purposes with the HC2 test, results of the CLART assay were considered as positive if one of the following 13 HR-HPV types (CLART-13 HR-HPV) was present: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. In addition, the evaluation was performed also considering 17 (probably) HR-HPV types (CLART-17 HR-HPV; HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) [Bernard et al., 2010].

The sensitivity, specificity, NPV, and positive predictive value (PPV) were calculated using 2×2

TABLE I. Characteristics of the Studied Cervical Samples

	n (%)
Cytological results (n = 425)	
Normal	63 (14.8)
ASCUS	111 (26.1)
LSIL	127 (29.9)
HSIL	118 (27.8)
SCC	6 (1.4)
Histological results (n = 425)	
Normal	95 (22.4)
CIN1	63 (14.8)
CIN2	118 (27.8)
CIN3	98 (23.1)
SCC	8 (1.9)
ND	43 (10.1)
Clinical diagnosis (cytology + biopsy; n = 405)	
≤CIN1	178 (44.0)
≥CIN2	227 (56.0)
ND	20
CLART-13 HR (n = 425)	
Positive	268 (63.1)
Negative	157 (36.9)
CLART-17 HR (n = 425)	
Positive	275 (64.7)
Negative	150 (35.3)
HC2 (n = 425)	
Positive	274 (64.5)
Negative	151 (35.5)

ASCUS, atypical squamous cells of undeterminated significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; CIN1, cervical intraepithelial neoplasia grade 1; CIN2, cervical intraepithelial neoplasia grade 2; CIN3, cervical intraepithelial neoplasia grade 3; ND, no determined.

contingency tables with 95% confidence intervals (95% CI). All *P*-values were obtained using the Fisher's exact test or McNemar χ^2 for comparison of matched-pair samples. *P*-values <0.05 were considered statistically significant. Agreement between assays was assessed by Cohen's kappa (κ) statistics. All analyses were conducted using the SPSS (*Statistical Package for the Social Sciences*; SPSS, Inc., Chicago, IL) version 16.0 software.

RESULTS

A total of 425 cervical samples from women aged 25–63 years old (mean age: 34.4 ± 10.0 years; median age: 35 years old) were studied. From these, a subset of 405 samples was available for clinical evaluation of the CLART assay (Table I). Results of HPV DNA assays and clinical diagnosis (based on cytology and biopsy) were shown in Table I. Overall, HR-HPV types were detected in 268 cases (63.1%) with CLART-13 HR-HPV, in 275 cases (64.7%) with CLART-17 HR-HPV, and in 274 (64.5%) with the HC2 test (Table I).

Clinical performance of the two tests for CIN grade 2 or worse (≥CIN2) was very good and comparable, with sensitivities of 96% for CLART-13 HR-HPV and HC2, and 96.9% for CLART-17 HR-HPV. The specificity and NPV of all the tests were also comparable (Table II). No significant statistical differences were found among assays, in terms of sensitivity, specificity, and NPV ($P = 0.000$).

Overall, agreement between the CLART and the HC2 was very good ($\kappa = 0.969$, 95% CI: 0.941–0.969; concordance: 98.6% ± 1.2 for 13 HR-HPV, and $\kappa = 0.974$, 95% CI: 0.941–0.988; concordance: 98.8% ± 1.1 for 17 HR-HPV; $P = 0.000$). Compared with the HC2 test, the CLART-13 HR-HPV assay showed a sensitivity, specificity, PPV, and NPV of 97.8% (95% CI: 96.8–97.8), 100% (95% CI: 98.1–100), 100% (95% CI: 98.9–100), and 96.2% (95% CI: 94.4–96.2), respectively (Table III). For 17 HR-HPV the CLART assay showed a sensitivity, specificity, PPV, and NPV of 99.3% (95% CI: 98.1–99.8), 98% (95% CI: 95.9–98.9), 98.9% (95% CI: 97.7–99.4), and 98.7% (95% CI: 96.5–99.6), respectively (Table III).

Discordant results were observed in six samples, which were positive by the HC2 test and negative by the CLART assay for the same 13 HR-HPV types. Considering CLART-17 HR-HPV, two samples were negative and four were positive (for HPV 53, 66, and 82). Further testing by the PapilloCheck revealed a total concordance with the results obtained by CLART-17 HR-HPV (Table IV).

TABLE II. Clinical Performance for ≥CIN2 Based in Pap Smear and Biopsy Diagnosis of 405 Samples

	Clinical diagnosis					
	≤CIN1 (n = 178)	≥CIN2 (n = 227)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
CLART-13 HR						
P	47	218	96.0 (92.6–97.9)	73.6 (66.7–79.5)	82.2 (79.4–82.7)	93.6 (93.0–94.2)
N	131	9				
Positivity (%)	26.4	96.0				
CLART-17 HR						
P	50	220	96.9 (93.8–98.5)	71.9 (64.9–78.0)	81.5 (79.9–83.1)	94.8 (94.3–95.3)
N	128	7				
Positivity (%)	28.1	96.9				
HC2						
P	51	218	96.0 (92.6–97.9)	71.4 (64.3–77.5)	81.0 (79.4–82.7)	93.4 (92.8–94.0)
N	127	9				
Positivity (%)	28.7	96.0				

≤CIN1, cervical intraepithelial neoplasia grade 1 or less; ≥CIN2, cervical intraepithelial neoplasia grade 2 or worse; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; P, HPV positive; N, HPV negative.

TABLE III. Analytical Performance of CLART and HC2 in 425 Samples

	HC2		Concordance	κ	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	P	N	% ± SD	(95% CI)	(95% CI)	(95% CI)	(95% CI)	(95% CI)
CLART-13 HR								
P	268	0	98.6 ± 1.2	0.969	97.8	100	100	96.2
N	6	151		(0.941–0.969)	(96.8–97.8)	(98.1–100)	(98.9–100)	(94.4–96.2)
CLART-17 HR								
P	272	3	98.8 ± 1.1	0.974	99.3	98.0	98.9	98.7
N	2	148		(0.941–0.988)	(98.1–99.8)	(95.9–98.9)	(97.7–99.4)	(96.5–99.6)

PPV, positive predictive value; NPV, negative predictive value; κ, agreement; CI, confidence interval; SD, standard deviation; P, HPV positive; N, HPV negative.

In addition, the CLART assay was evaluated for reproducibility (n = 75). The assay showed a concordance of 98.7% (±2.7%) and an agreement of 0.972 (95% CI: 0.919–1) for the samples tested (data not shown).

DISCUSSION

Recent studies [Castle et al., 2005; Lai et al., 2007; Cuzick et al., 2008] have shown that detection of HR-HPV, in particular of HPV 16 and 18, can be an important aspect to consider in cervical cancer screening programs and algorithms. Since most HR-HPV infections can clear spontaneously, a clinically useful HPV test needs to have an optimal clinical sensitivity and specificity in order to effectively detect CIN grade 2 or worse (≥CIN2). Tests with a better sensitivity than that of the HC2 will detect a large number of latent infections that are clinically irrelevant and lead to an overtreatment of women with transient HPV infections [Snijders et al., 2003; Meijer et al., 2009].

In this study, the performance of a new commercially available CE-marked genotyping assay (CLART Human Papillomavirus 2) that allows the detection and genotyping of 35 HPV types was compared with the HC2 test for the same 13 HR-HPV types, as well as for 17 HR-HPV types, which includes HPV 53, 66, 73, and 82. Moreover, the clinical performance was evaluated on a subset of 405 samples with histological results.

Both the CLART-13 HR-HPV and the HC2 assays showed an identical clinical performance, with a clinical sensitivity of 96%. For 17 HR-HPV types, the clinical sensitivity of the CLART assay was 96.9%. Regarding the specificity and the NPV, the results of the CLART and the HC2 were also very similar. In large population-based trials, the HC2 test had a specificity of ≥95% [Meijer et al., 2009]. In this study, all the assays had a positive rate ranging from 26.4% to 28.7% in the control group (CIN grade 1 or less), which can explain the lower clinical specificity observed (71.9% for CLART-17 HR-HPV, 73.6% for CLART-13 HR-HPV, and 71.4% for the HC2), compared to ≥95% specificity in a screening population [Ronco et al., 2008; Meijer et al., 2009]. Similar data were reported by using other tests [Halfon et al., 2010; Schopp et al., 2010].

Analytical performance of the CLART assay showed highly comparable outcomes, with very good values of sensitivity, specificity, PPV, NPV, and concordance compared with the HC2 test. In addition, the CLART assay reproducibility was very good (κ = 0.972).

HPV discrepancies between the CLART assay and the HC2 test were found in six cases. Analysis of these cases by the PapilloCheck system indicated a total concordance with the results obtained by the CLART assay.

As explained elsewhere [Snijders et al., 2003], it is necessary to distinguish between clinical and analytical sensitivity. Whether a method with a higher analytical sensitivity would result in a better performance in terms of clinical sensitivity and specificity is still unknown.

In conclusion, the CLART Human Papillomavirus 2 assay showed an excellent performance, very similar to that of the HC2 test, which has a defined clinically cut-off. The CLART assay is efficient, sensitive, and reproducible, Furthermore, this assay has the advantage of detecting and genotyping 35 HPV types, in single or multiple infections, by a single test, which can be important for the individual risk stratification of women found to be infected persistently by HR-HPV types, as well as for the population studies required for vaccination trials and for monitoring the efficacy of HPV vaccines.

TABLE IV. Discordant Results Between CLART and HC2

Case	CLART-13 HR-HPV	CLART-17 HR-HPV	HC2	PapilloCheck	Cytology/biopsy
1	N	HPV 66	P	HPV 66	CIN1
2	N	N	P	N	CIN1
3	N	N	P	N	Normal
4	N	HPV 82	P	HPV 82	CIN1
5	N	HPV 53	P	HPV 53	ASCUS
6	N	HPV 66	P	HPV 66	ASCUS

P, HPV positive; N, HPV negative; ASCUS, atypical squamous cells of undeterminated significance; CIN1, cervical intraepithelial neoplasia grade 1.

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CHAPTER *5*

Ana Gradíssimo, Nuno Verdasca, Ângela Pista

Contributions of the Authors:

Ana Gradíssimo was responsible for the research, writing and organization of this paper. She was also responsible for all the data analysis, including figures and tables, as well as laboratory work, namely DNA extraction, HPV genotyping, Hybrid Capture® 2 High-Risk HPV DNA Test (HC2) and Cobas® 4800 HPV tests.

Nuno Verdasca contributed with proofreading and image rearrangements. He also conducted laboratory work, including DNA extraction, HPV genotyping, Abbott RealT*im*e High-Risk (HR) HPV and APTIMA® HPV tests.

Ângela Pista contributed with scientific guidance and also performed laboratory work, namely HC2 and Cobas® 4800 HPV tests.

ORIGINAL ARTICLE

Evaluation of several human papillomavirus tests for use in clinical practice

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Running Title: HPV testing evaluation for clinical practice

ABSTRACT

Persistent infection by high-risk human papillomavirus (HR-HPV) is the necessary cause of cervical cancer. The use of HPV detection in cervical screening programs can improve the ability to identify women at risk of cervical cancer. Therefore, the use of adequate methods is essential. The aim of this study was to evaluate the clinical performance of different HPV tests in comparison with the Hybrid Capture® 2 High-Risk HPV DNA Test (HC2), using a clinical cut-off of cervical intraepithelial neoplasia grade 2 or worse. Considering the 731 samples included in this study, HR-HPV positivity rates were similar among DNA tests (HC2: 71.7%; CLART: 69.6%; Cobas® 4800 HPV: 69.8%; Abbott RealTi*m*e High-Risk HPV: 68.2%). Overall, the agreement between DNA tests was comparable (> 0.860). For the HPV RNA testing, the positivity rate was lower compared to DNA testing (APTIMA® HPV: 65.4%; NucliSENS® EasyQ® HPV: 49.5%). HPV DNA tests showed a clinical sensitivity over 93%, while RNA tests were less sensitive to detect cervical disease (79.4%). However, RNA testing increased the clinical specificity (77.2%) when compared to DNA testing (49.2%). Moreover, specificity increased greatly for each assay evaluated when only HPV 16 and 18 detection was considered (> 80.0%). In conclusion, the HPV DNA tests under evaluation may be considered efficient, sensitive and reproducible, evidencing similar performance to HC2 test. RNA tests revealed as more specific, improving the management of HPV-infected women, and may discriminate the clinically relevant HPV infections in routine practice.

Keywords: Human papillomavirus, genotyping, DNA testing, clinical practice, cervical cancer.

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INTRODUCTION

Epidemiological studies have established that certain human papillomavirus (HPV) genotypes are aetiologically related to cervical cancer development [1-3]. More than 120 HPV genotypes have been identified, of which, approximately 40 can infect the human genital tract mucosa. According to the oncogenic potential, these HPV genotypes can be classified into high-risk (HR), associated with high-grade cervical lesions and cervical cancer, and low-risk (LR), found mainly in benign lesions [4,5]. Most infections will clear spontaneously, but persistent infection with HR-HPV genotypes has been considered a strong predictor of the development of high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer [6,7].

In areas where the Papanicolau (Pap) smear is the primary screening method, the unnoticed development of high-grade cervical lesions may be attributed to the low reproducibility and sensitivity of the Pap test [8]. Several studies have shown that the combined use of cytology and HPV DNA testing in women above 30 years old can improve the sensitivity and the negative predictive value (NPV) of routine screening. HPV DNA testing may also provide reassurance to extended re-screening intervals, and can be cost-effective in the prevention of the development of high-grade cervical lesions in women with cytological abnormalities. Several HPV assays with various levels of sensitivity and specificity have been made available lately. However, clinical validation is required prior to the use as a "stand-alone" method in cervical cancer screening programs. As an example, tests with significantly higher sensitivity than the Hybrid Capture® 2 High-Risk HPV DNA Test (HC2; Qiagen, Gaithersburg, MD, USA) for the detection of HPV DNA could detect latent infections that are clinically irrelevant, which may lead to overtreatment of women [9,10]. Recently, an assay based on PCR and hybridization, the CLART® Papillomavirus Humano 2 (CLART; Genomica, Madrid, Spain) was developed for the detection and genotyping of 35 HPV genotypes (20 HR-HPV and 15 LR-HPV), either in single or multiple infections.

Some studies on clinical validation of HPV DNA testing have gather evidences that a single positive result either for HPV 16 or 18 provides a high predictive value for CIN grade 2 or worse (≥CIN2) [11,12]. These results supported the clinical importance of HPV 16 and/or 18 infections and raised the emergence of HPV DNA screening tests including the specific identification of HPV 16 and 18, which are referred to as 'HR-HPV DNA detection assays with concurrent or reflex HPV 16 and HPV 18 genotyping' [13]. Two of these commercial systems are fully automated, and they are based in a multiplex real-time PCR methodology that identifies HPV 16 and 18 with concurrent detection of twelve other HPV genotypes simultaneously (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68): the Cobas® 4800 HPV (Roche Molecular Systems, Inc, Pleasanton, CA, USA) and the Abbott RealTi*m*e High-Risk (HR) HPV (Abbott Molecular, Des Plaines, IL, USA).

Regarding HPV RNA testing, there are two commercial systems targeting mRNA detection: the APTIMA® HPV (Hologic Gen-Probe Incorporated, San Diego, CA, USA), and the NucliSENS® EasyQ® HPV (bioMérieux SA, Marcy l'Etoile, France). The APTIMA® HPV assay detects qualitatively the presence of mRNA of at least one of 14 HR-HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). This mRNA assay, while increasing the specificity and the positive predictive value (PPV), can also achieve sensitivity and NPV values similar to HPV DNA testing, so that it may be used in routine cervical cancer screening programs as an adjunct to the widely used cytology approach [14-16]. The NucliSENS® EasyQ® HPV semi-quantifies the over-expression of E6 and E7 genes for five HR-HPV genotypes (HPV 16, 18, 31, 33, and 45). The higher specificity of this mRNA assay would reduce false positives, v.g. it excludes transient infections, so that this assay could be used as a triage test for HPV DNA-positive women, reducing the colposcopy referral in cytological abnormalities and therefore improving patient management [17,18].

The aim of this study was to evaluate the performance of different HPV tests [CLART® Papillomavirus Humano 2 (CLART; Genomica, Madrid, Spain); Cobas® 4800 HPV (Roche Molecular Systems, Inc, Pleasanton, CA, USA); Abbott RealTi*m*e High-Risk (HR) HPV (Abbott Molecular, Des Plaines, IL, USA); APTIMA® HPV (Hologic Gen-Probe Incorporated, San Diego, CA, USA); NucliSENS® EasyQ® HPV (bioMérieux SA, Marcy l'Etoile, France)] in comparison to the clinically validated Hybrid Capture® 2 High-Risk HPV DNA Test (HC2; Qiagen, Gaithersburg, MD, USA) test in cervical samples for which histological results were available. The clinical performance was also evaluated by histological grade, using a clinical cut-off of CIN grade 2 or worse (≥ CIN2).

To simplify the reading of the text, from this section on, manufacturers address will not be displayed.

MATERIAL AND METHODS

Study Design

The study population comprised 731 archived cervical DNA samples selected from an enlarged longitudinal study (Chapter 3), with the following selection criteria: (1) age range of 18 to 65 years; (2) sexually active life; and (3) referral from a gynecological clinic. Criteria for exclusion were (1) pregnancy; (2) history of atypical cytology, CIN lesions or treatment for cervical disease in the prior 12 months; and (3) having had a hysterectomy. DNA samples were obtained from residual liquid-based cytology (LBC) as previously described (Chapter 3).

The final diagnosis, based on histological examination of biopsy samples obtained at colposcopy, was made available for the purposes of the present evaluation. Table 1 summarizes the characteristics of women from whom specimens were obtained. As suggested by Wentzensen *et al.*

[19] and according to histology, 386 out of the 731 samples (52.8%) were considered to have CIN grade 1 or less (≤ CIN1, regarded as controls), while 345 (47.2%) were diagnosed as CIN grade 2 or worse (≥ CIN2, regarded as cases).

Laboratory Methods

HPV DNA was isolated from 1 ml of cellular suspension using the automated extraction system NucliSENS® easyMAG® (bioMérieux, Boxtel, Netherlands), as specified in the manufacturer's instructions. Nucleic acids were eluted in a final volume of 100 µl and stored at -20ºC until use for PCR analysis. This DNA extraction was used in CLART and NucliSENS® EasyQ® HPV assays.

All samples were tested by HC2, CLART and NucliSENS® EasyQ® HPV assays. Furthermore, 725 (99.2%) were tested by both Cobas® 4800 HPV and Abbott RealTi*m*e HR HPV assays, and 719 (98.4%) samples were tested by APTIMA® HPV assay. Each HPV test was carried out independently of each other according to the manufacturer's instructions.

Table 1: Characteristics of study samples

Characteristic, n (%)	Study Sample (n=731)
Age of HPV-infected women, mean (SD)	35.0 (10.4)
Age distribution in years	
18-19	6 (0.8)
20-24	88 (12.0)
25-29	170 (23.3)
30-39	267 (36.5)
40-49	123 (16.8)
50-59	48 (6.6)
60-65	29 (3.9)
Cytology results	
NILM	216 (29.4)
ASC-US	133 (18.2)
LSIL	178 (24.4)
HSIL	188 (25.7)
ICC	16 (2.2)
Histological results	
Normal	263 (36.0)
CIN1	128 (17.5)
CIN2	193 (26.4)
CIN3	139 (19.0)
ICC	8 (1.1)
Clinical diagnosis (cytology + biopsy)	
≤ CIN1	386 (52.8)
≥ CIN2	345 (47.2)
≤ CIN2	578 (79.1)
≥ CIN3	153 (20.9)

NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, invasive cervical cancer; CIN, cervical intraepithelial neoplasia grade 1-3.

Hybrid Capture® 2 High-Risk HPV DNA Test (HC2)

The HC2 test is a sandwich capture molecular hybridization assay that uses a signal amplification detection method based on chemiluminescence to detect 13 HR-HPV genotypes (HPV

16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 68). The resultant DNA:RNA hybrids are captured on a microplate, and the emitted light is measured in a luminometer as relative light units (RLU). Samples were considered as positive if the ratio RLU/cut-off was ≥ 1.0 (equivalent to 1.0 pg HPV DNA/ml). In the present study, cut-off values between 1 and 2.5 were confirmed by retesting with HC2 before being considered as positives.

CLART® Papillomavirus Humano 2 (CLART)

The CLART test uses biotinylated consensus primers that amplify a 450 bp fragment within the HPV L1 region. To avoid false-negative results, co-amplification of an 892 bp region of the human housekeeping gene CFTR (*cystic fibrosis transmembrane conductance regulator*) and of a 1,202 bp fragment of a transformed plasmid is performed, to ensure DNA extraction adequacy and PCR efficiency. HPV genotyping is performed by hybridization in a low-density microarray containing triplicate DNA probes specific to 35 genotypes (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89). Semi-quantitative results were obtained in an automatic reader. A detailed evaluation of the clinical performance of this methodology is presented in Paper II (Chapter 4).

Cobas® 4800 HPV

The Cobas® 4800 HPV test is a fully automated qualitative multiplex assay that provides HPV 16 and 18 genotyping, while concurrently detects other 12 HR-HPV genotypes in a pooled result (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), either in single or multiple infections. This assay uses the human β-globin gene as an internal control to assess specimen quality. The system comprehends a sample preparation robotic platform (cobas x 480 instrument; DNA extraction and real-time PCR platting) and a real-time PCR apparatus (cobas z 480 analyser) performing up to 96 tests per run, for the amplification and detection of L1 gene.

Abbott RealTi*m*e High-Risk (HR) HPV

The Abbott RealTi*m*e HR HPV test is a qualitative multiplex real-time PCR performed in a fully automated platform (m2000 system) for the detection of HPV 16 and 18, along with 12 other HR-HPV genotypes in a pooled result (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), either in single or multiple infections. The test comprehends homogeneous target amplification of L1 gene using single-stranded linear probes, performing up to 96 tests per run. In addition, the endogenous human β-globin gene is used as an internal control for sample validity.

APTIMA® HPV

The APTIMA® HPV test is a transcription-mediated amplification-based assay, which allows the detection of E6/E7 mRNA transcripts of 14 HR-HPV genotypes in a pooled result (HPV

16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and includes an unspecified non-infectious RNA transcript internal control to ensure sample adequacy (details on the internal control are Hologic confidential). The test is based on mRNA target capture by specific oligomers linked to magnetic microparticles followed by a transcription-mediated amplification and by production of labeled RNA:DNA hybrids emitting chemiluminescence reported as a signal-to-cutoff ratio, in which ≥ 0.5 determines positivity.

Hologic Gen-Probe Incorporated provides platforms including several levels of automation, from a semi-automated Direct-Tube Sampling (DTS; used in the present study) to TIGRIS DTS (fully automated).

NucliSENS® EasyQ® HPV

The NucliSENS® EasyQ® HPV is a nucleic acid sequence-based amplification (NASBA) system based on isothermal amplification of E6/E7 mRNA from high-risk HPV 16, 18, 31, 33, and 45, through different molecular beacon probes. Integrity of RNA in the specimen is confirmed by using a probe directed against the human U1 small nuclear ribonucleoprotein-specific mRNA (U1A). The use of two fluorescent dyes, 6-carboxy fluorescein (6-FAM) for HPV genotypes 16, 31 and 33, and 6-carboxy X-rhodamine (6-ROX) for HPV genotypes 18 and 45, and for U1A protein, allows simultaneous duplex amplification. The detailed evaluation of the clinical performance of this methodology is presented in Paper III (Chapter 6).

Statistical Methods

The histological diagnosis defining cervical intraepithelial neoplasia grade 2 or worse (≥CIN2) as a disease endpoint was used to evaluate the clinical performance of the different tests. Eligible samples corresponded to an opportunistic screening population, with a clinical diagnosis suggestive of HPV infection. Although not a routine screening population, the advantage was a broad range of outcomes and a high disease rate, which would enable accurate evaluation of sensitivity and specificity in a relatively small sample.

The HC2 test was used as the reference standard method as it encompasses a clinically defined cut-off value. Considering data harmonization, a CLART-positive result was considered: (1) when one (or more) of the following 13 HR-HPV genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) (CLART-13) was present for further comparison with HC2 and APTIMA® HPV; (2) when one (or more) of the following 5 HR-HPV genotypes (HPV 16, 18, 31, 33, and 45) (CLART-5) was present for further comparison with NucliSENS® EasyQ® HPV; and (3) a HPV 16 and/or 18-positive reflex result was considered when HPV 16 or 18 were detected concurrently with one (or more) of the following 12 HR-HPV genotypes (HPV 31, 33, 35, 39, 45, 51, 52 56, 58, 59, 66, and 68) (CLART-16/18) for further comparison with Cobas® 4800 HPV and Abbott RealTi*m*e HR HPV.

The sensitivity, specificity, NPV, and PPV of each assay were calculated using 2 x 2 contingency tables with 95% confidence intervals (95% CI). Agreement between assays was assessed by Cohen's kappa (κ) statistics, where values ranging from 0.00 to 0.20 were considered as indicating poor agreement; 0.21 to 0.40 indicating fair agreement; 0.41 to 0.60 indicating moderate agreement; 0.61 to 0.80 indicating good agreement; and 0.81 to 1.00 indicating very good to perfect agreement. All P values were obtained using the Fisher's exact test or McNemar $\chi 2$ for comparison of matched-pair samples. A two-sided P values < 0.05 was considered statistically significant. All analyses were performed by using the IBM SPSS Statistics version 20.0 (IBM Corporation Inc., Armonk, NY, USA).

RESULTS

HPV testing results are presented in Table 2.

Table 2: HPV testing results

HPV Test, n (%)	Study Sample (n=731)
HC2	
Negative	207 (28.3)
Positive	524 (71.7)
CLART	
Negative	222 (30.4)
CLART-13 Positive	502 (68.7)
CLART-5 Positive	440 (60.2)
CLART-16/18 Positive	299 (40.9)
Cobas® 4800 HPV	
Negative	219 (30.2)
Positive	506 (69.8)
HR-Positive	210 (29.0)
16/18-Positive	296 (40.8)
Data missing*	6
Abbott RealTi*m*e HR HPV	
Negative	230 (31.7)
Positive	495 (68.2)
HR-Positive	209 (28.8)
16/18-Positive	286 (39.4)
Data missing*	6
APTIMA® HPV	
Negative	249 (34.6)
Positive	470 (65.4)
Data missing*	12
NucliSENS® EasyQ® HPV	
Negative	369 (50.5)
Positive	362 (49.5)
16/18-Positive	256 (35.0)

HR, high-risk; * Not determined, unknown.

Overall, HR-HPV genotypes were detected in 524 cases (71.7%) using the reference test, HC2, which provided the highest positivity rate. CLART test positivity rate was 69.6% (509/731; including HPV 68 in addition to the 13 HR-HPV genotypes detected by HC2). Cobas® 4800 HPV test positivity rate was 69.8% (509/725), whereas 40.8% (296/725) corresponded to HPV 16 and 18

infections. Abbott RealTi*m*e HR HPV positivity rate was 68.2% (495/725), of which 39.4% (286/725) were HPV 16 and 18 infections. Regarding HPV RNA testing, APTIMA® HPV tested positive for 65.4% (470/719) of the cases, and NucliSENS® EasyQ® HPV detected 362 positive results (49.5%), of which 35.0% (256/731) corresponded to HPV 16 and 18 infections (Table 2).

The performance of the different tests for disease cases (≥ CIN2) was in accordance with histological findings and comparable sensitivities could be observed: HC2, 95.1% (95% CI: 92.3-96.9); CLART, 94.5% (95% CI: 91.6-96.5) (94.2% for CLART-13; 88.1% for CLART-5; and 65.8% for CLART-16/18); Cobas® 4800 HPV, 94.7% (95% CI: 91.9-96.7) (66.2% for Cobas-16/18); Abbott RealTi*m*e HR HPV, 93.6% (95% CI: 90.5-95.7) (65.0% for Abbott-16/18); APTIMA® HPV, 93.5% (95% CI: 90.4-95.7); and NucliSENS® EasyQ® HPV, 79.4% (95% CI: 74.8-83.4) (61.2% for NucliSENS-16/18) (Table 3).

The specificity of all tests was also comparable, ranging from 49.2% (95% CI: 44.3-54.2) for HC2 to 60.1% (95% CI: 55.0-64.9) for APTIMA® HPV test. NucliSENS® EasyQ® HPV was the test evidencing the highest specificity (77.2%; 95% CI: 72.8-81.1). For each assay, specificity increased greatly when only HPV 16 and 18 detection was considered (> 80.0%). No significant statistical differences were found among the different assays, in terms of sensitivity, specificity, and NPV (P = 0.662) (Fig.1).

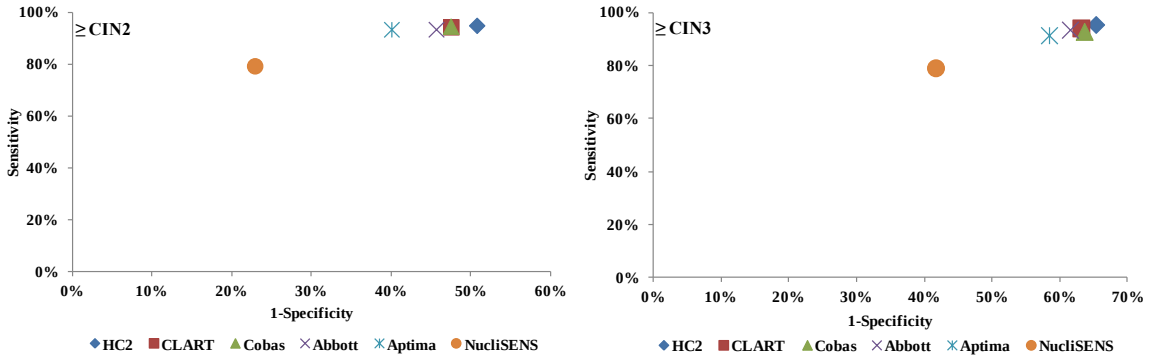

Fig.1: ROC Curves for CIN grade 2 or worse (≥ CIN2) disease endpoint and CIN grade 3 or worse (≥ CIN3) disease endpoint.

Overall, the agreement between the HC2 and CLART-13 was considerable (κ = 0.869, 95% CI: 0.830-0.909; concordance: 94.5% ± 1.7), as well as between the assays HC2 and Cobas® 4800 HPV (κ = 0.887, 95% CI: 0.850-0.924; concordance: 95.3% ± 3.6), and between HC2 and Abbott RealTi*m*e HR HPV (κ = 0.866, 95% CI: 0.826-0.906; concordance: 94.3% ± 1.7).

Table 3: Clinical performance of different HPV tests for ≥ CIN2 based in Pap smear and biopsy diagnosis of 731 samples

	Clinical Diagnosis					
	≤ CIN1 (n = 386)	≥ CIN2 (n = 345)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
HC2 (n = 731)						
P	196	328	95.1	49.2	62.6	91.8
N	190	17	(92.3 – 96.9)	(44.3-54.2)	(58.3-66.8)	(87.2-95.1)
Positivity (%)	50.8	95.1				
CLART (n = 731)						
P	183	326	94.5	52.6	64.1	91.4
N	203	19	(91.6 – 96.5)	(47.6-57.5)	(59.7-68.2)	(86.9-94.8)
Positivity (%)	47.4	94.5				
CLART-13 (n = 731)						
P	177	325	94.2	54.2	64.7	91.3
N	209	20	(91.2-96.2)	(49.2-59.2)	(60.4-68.9)	(86.8-94.6)
Positivity (%)	45.8	94.2				
CLART-5 (n = 731)						
P	136	304	88.1	64.8	69.1	85.9
N	250	41	(84.3-91.1)	(59.9-69.4)	(64.5-73.4)	(81.4-89.7)
Positivity (%)	35.2	88.1				
CLART-16/18 (n = 731)						
P	72	227	65.8	81.3	75.9	72.7
N	314	118	(60.6-70.6)	(77.2-84.9)	(70.7-80.7)	(68.2-76.8)
Positivity (%)	18.7	65.8				
Cobas® 4800 HPV (n = 725)						
P	181	325	94.7	52.6	64.2	91.8
N	201	18	(91.9 – 96.7)	(47.6-57.6)	(59.9-68.4)	(87.3-95.1)
Positivity (%)	47.4	94.7				
Cobas-16/18 (n = 725)						
P	69	227	66.2	81.9	76.7	73.0
N	313	116	(61.0-71.0)	(77.8-85.5)	(71.4-81.4)	(68.5-77.1)
Positivity (%)	18.1	66.2				
Abbott RealTi*m*e HR HPV (n = 725)						
P	174	321	93.6	54.5	64.9	90.4
N	208	22	(90.5 – 95.7)	(49.4-59.4)	(60.5-69.1)	(85.9-93.9)
Positivity (%)	45.5	93.6				
Abbott-16/18 (n = 725)						
P	63	223	65.0	83.5	78.0	72.7
N	319	120	(59.8-69.9)	(79.5-86.9)	(72.7-82.6)	(68.2-76.8)
Positivity (%)	16.5	65.0				
APTIMA® HPV (n = 719)						
P	151	319	93.5	60.1	67.9	91.2
N	227	22	(90.4 – 95.7)	(55.0-64.9)	(63.4-72.1)	(86.9-94.4)
Positivity (%)	39.9	93.5				
NucliSENS® EasyQ® HPV (n = 731)						
P	88	274	79.4	77.2	75.7	80.8
N	298	71	(74.8 – 83.4)	(72.8-81.1)	(70.9-80.0)	(76.4-84.7)
Positivity (%)	22.8	79.4				
NucliSENS-16/18 (n = 731)						
P	45	211	61.2	88.3	82.4	71.8
N	341	134	(55.9-66.2)	(84.8-91.2)	(77.2-86.9)	(67.5-75.8)
Positivity (%)	11.7	61.2				

≤ CIN1, cervical intraepithelial neoplasia grade 1 or less; ≥ CIN2, cervical intraepithelial neoplasia grade 2 or worse; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; P, HPV positive; N, HPV negative.

When comparing HC2 with HPV DNA tests providing a positive result for fewer genotypes, the agreement decreased greatly, ranging between $\kappa = 0.712$ and $\kappa = 0.251$ (data not shown). Regarding the comparison between HC2 with HPV RNA tests, a considerable agreement could be observed with APTIMA® HPV ($\kappa = 0.779$; 95% CI: 0.730-0.828; concordance: 90.4% ± 2.1), but it was less obvious with NucliSENS® EasyQ® HPV for which a moderate kappa value was determined ($\kappa = 0.542$; 95% CI: 0.488-0.597; concordance: 62.5% ± 3.5).

Not Published.

Table 4: Clinical performance of different HPV tests for ≥ CIN3 based in Pap smear and biopsy diagnosis of 731 samples

	Clinical Diagnosis					
	≤ CIN2 (n = 578)	≥ CIN3 (n = 153)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
HC2 (n = 731)						
P	378	146	95.4	34.6	27.9	96.6
N	200	7	(90.9 – 97.8)	(30.8-38.6)	(24.1-31.9)	(93.2-98.6)
Positivity (%)	65.4	95.4				
CLART (n = 731)						
P	365	144	94.1	36.8	28.3	95.9
N	213	9	(89.2 – 96.9)	(33.0-40.9)	(24.4-32.4)	(92.4-98.1)
Positivity (%)	63.2	94.1				
CLART-13 (n = 731)						
P	359	143	93.5	37.9	28.5	95.6
N	219	10	(88.4-96.4)	(34.0-41.9)	(24.6-32.6)	(92.1-97.9)
Positivity (%)	62.1	93.5				
CLART-5 (n = 731)						
P	305	135	88.2	47.2	30.7	93.8
N	273	18	(82.2-92.4)	(43.2-51.3)	(26.4-35.2)	(90.4-96.3)
Positivity (%)	52.8	88.2				
CLART-16/18 (n = 731)						
P	190	109	71.2	67.1	36.4	89.8
N	388	44	(63.6-77.8)	(63.2-70.8)	(31.0-42.2)	(86.6-92.5)
Positivity (%)	32.9	71.2				
Cobas® 4800 HPV (n = 725)						
P	364	142	92.8	36.4	28.1	95.0
N	208	11	(87.6 – 95.9)	(32.5-40.4)	(24.2-32.2)	(91.2-97.5)
Positivity (%)	63.6	92.8				
Cobas-16/18 (n = 725)						
P	184	112	73.2	67.8	37.8	90.4
N	388	41	(65.7-79.6)	(63.9-71.5)	(32.3-43.6)	(87.3-93.1)
Positivity (%)	32.2	73.2				
Abbott RealTi*m*e HR HPV (n = 725)						
P	352	143	93.5	38.5	28.9	95.6
N	220	10	(88.4 – 96.1)	(34.6-42.5)	(24.9-33.1)	(92.1-97.9)
Positivity (%)	61.5	93.5				
Abbott-16/18 (n = 725)						
P	175	111	72.6	69.4	38.8	90.4
N	397	42	(65.0-79.0)	(65.5-73.0)	(33.1-44.7)	(87.3-93.0)
Positivity (%)	30.6	72.6				
APTIMA® HPV (n = 719)						
P	332	138	91.4	41.5	29.4	94.5
N	236	13	(85.8 – 94.9)	(37.6-45.7)	(25.3-33.7)	(91.2-97.2)
Positivity (%)	58.5	91.4				
NucliSENS® EasyQ® HPV (n = 731)						
P	241	121	79.1	58.3	33.4	91.3
N	337	32	(72.0 – 84.8)	(54.2-62.3)	(28.6-38.5)	(88.0-94.0)
Positivity (%)	41.7	79.1				
NucliSENS-16/18 (n = 731)						
P	154	102	66.7	73.4	39.8	89.3
N	424	51	(58.9-73.7)	(69.6-76.8)	(33.8-46.1)	(86.1-91.9)
Positivity (%)	26.6	66.7				

≤ CIN2, cervical intraepithelial neoplasia grade 2 or less; ≥ CIN3, cervical intraepithelial neoplasia grade 3 or worse; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; P, HPV positive; N, HPV negative.

When evaluating the clinical performance of the several tests for CIN grade 3 or worse (≥CIN3) cases, some differences could be observed towards the results obtained for ≥ CIN2 disease endpoint. The overall sensitivity of all tests was maintained (HC2: 95.4%; CLART: 94.1%; Cobas® 4800 HPV: 92.8%; Abbott RealTi*m*e HR HPV: 93.5%; APTIMA® HPV: 91.4%; NucliSENS®

EasyQ® HPV: 79.1%), but a corresponding decrease in specificity could be observed (Table 4) (Fig.1).

DISCUSSION

HR-HPV infection is a necessary step in cervical cancer development, and consequently, HPV DNA detection constitutes a promising target for primary screening of this potentially fatal neoplasia [20-23]. In general, HPV DNA testing is considered as having higher sensitivity and lesser specificity than cytology [24]. However, HPV DNA tests have been validated only for women older than 30 years, as an adjunct to cytological screening [14], and their applicability to screening depends on intrinsic characteristics of each population. In the absence of a large evaluation of HPV DNA tests in the Portuguese population, it was considered imperative to determine their performance. Similarly, it was considered necessary to evaluate HPV mRNA tests on a Portuguese cervical sample collection, for which clinical histological findings were available. Moreover, considering that most HR-HPV infections clear spontaneously, the main question regarding the evaluation of any HPV test does not rely completely on its innate capacity for detecting HPV, but rather on its sensitivity and specificity towards the detection of high-grade cervical lesions (≥ CIN2) in order to be of real clinical usefulness. In fact, very sensitive tests will detect a large number of latent infections that are, for the most, clinically irrelevant, leading to overtreatment of women hosting transient HPV infections [9,10]. Also, it has been suggested that latent infections, putatively comprehend a low number of viral copies; accordingly, an HPV threshold below which HPV infection would not be clinically relevant should be established [25,26].

In current clinical practice, the most expanded and clinically validated HPV detection method is HC2, becoming considered as the reference standard against which all new HPV assays are evaluated [10], and for this reason, HC2 was chosen as the reference test in the present study. However, it is known that HC2 cross-reacts with non-oncogenic genotypes, thus potentially contributing to a reduction in specificity [27-29]. HC2 methodology is very laborious and time-consuming, as well as does not distinguish the HPV genotypes detected. Consequently, several new tests have became commercially available, including fully automated platforms that allow testing simultaneously large numbers of samples in about five hours. The aim of this study was to evaluate the performance of different HPV tests in comparison to HC2 test in cervical samples for which histological results were available .Furthermore, the clinical performance was evaluated by histological grade, using a clinical cut-off of CIN grade 2 or worse (≥ CIN2).

In the present study, HC2 and CLART-13 tests showed an identical clinical performance, with a clinical sensitivity of 95.0%. Regarding the specificity and the NPV, the results of the HC2 and CLART-13 tests were also similar (> 49.0% for specificity and > 91.0% for NPV). In the control

group (CIN grade 1 or less; ≤ CIN1), the positivity rate ranged from 39.9% to 50.8%, which can explain the low clinical specificity (49.2% for HC2; 54.2% for CLART-13), when compared to the specificity (70.5%) described by other authors [10,30] in screening populations where a positivity rate of 29.5% was detected, and 38.0% of specificity among another screening/disease population with 62.0% of HPV infection [16,31,32]. Compared to HC2, CLART test presents reproducibility characteristics that HC2 lacks [24]. Furthermore, this methodology is able to detect and identify 35 HPV genotypes within a single test, either in single or multiple infections, which is useful for individual risk stratification in women persistently infected by HR-HPV genotypes, as well as for epidemiological evaluations included in vaccination trials and for monitoring the efficacy of HPV vaccines [33].

CLART is better designed for epidemiological studies and HPV vaccination efficacy assessment, due to its technical approach of full genotyping of 35 HPV genotypes, either in single or multiple infections, within a single test. For large clinical settings might not be feasible, since it is more expensive when compared to other commercial systems evaluated through the present study Also, it has been described that HPV full genotyping has some technical issues that can produce loss of clinical sensitivity or reproducibility, namely primer competition in the amplification of the specific target [34].

The HPV infecting genotype is determinant for the carcinogenesis process. Worldwide, HPV 16 and 18 genotypes have been considered responsible for more than 70.0% of all cervical cancers and have been given prognostic value [11,35-37]. For this reason, the detection of HR-HPV, in particular of HPV 16 and 18, should be addressed in cervical cancer screening programs and algorithms, and it is expectable that the new generation of HPV tests discriminate these two oncogenic genotypes [11,12,14].

The Cobas® 4800 HPV test was designed for cervical cancer primary screening by the reflex genotyping of HPV 16 and 18 with concurrent detection of other 13 HR-HPV genotypes. This reflex genotyping that discriminates HPV 16 and 18 was considered very suitable for risk assessment in women with negative cytology but HR-HPV-positive [38]. In the present study, the Cobas® 4800 HPV test showed a high performance (higher sensitivity and specificity) for the disease endpoint ≥CIN2, when compared with HC2. In addition to specificity, sensitivity, and reproducibility, its fully automation provides a more suitable approach than HC2 for large scale screening studies. Moreover, within a single test, this methodology concurrently distinguishes HPV 16 and 18 from the remaining HR-HPV genotypes, which adds prognostic value to the HR-HPV infection diagnosis, due to the major contribution of these two genotypes in cervical cancer development.

The clinical sensitivity of Abbott RealTi*m*e HR HPV was very high (93.6%) across the different histological grades, especially for women with CIN2 or worse (≥ CIN2), but also high specificity, PPV, NPV and concordance (even when stratified by age). Compared to HC2, this methodology comprehends a fully automated system, which makes it suitable to large-scale cervical

cancer screening evaluations as proposed for Cobas® 4800 HPV test, as well as being more attractive than the manual procedure of HC2.

The APTIMA® HPV has been designed, and further approved, for screening of women over 21 years with ASC-US cytological diagnosis for reducing the need to colposcopy referral, and for assessing the presence or absence of HR-HPV in women 30 years or older as an adjunct to cytology. This methodology exhibited a high performance regarding sensitivity and NPV, 93.5% and 91.2%, respectively, with similar results when compared to HPV DNA testing, once again avoiding over-testing of HPV-infected women. Also, in a clinical trial of routine cervical cancer screening (CLEAR trial), APTIMA® HPV minimized about one quarter the false-positivity rate in comparison to HC2 [39]. However, this methodology does not discriminate the two most oncogenic HPV 16 and 18, in opposition to Cobas® 4800 HPV and Abbott RealTi*m*e HR HPV.

NucliSENS® EasyQ® HPV was launched prior to APTIMA® HPV, targeting only five HR-HPV genotypes, with the purpose of identifying the over expression of E6 and E7 mRNA in persistent infections. This methodology presented the highest specificity from all the tests under evaluation, being suitable for triage of HPV-infected women. Despite the fact that they are both HPV mRNA tests, they do not display the same applicability in a routine clinical setting. The NucliSENS® EasyQ® HPV is far more specific and can be of value during later stages of infection as a reflex test, namely in ASC-US or LSIL cytological diagnosis to estimate their risk of progression, as evaluated elsewhere [18].

In conclusion, and despite the difficulties of comparing the clinical performance of different HPV tests (comprehending diversified HPV genotypes), all the methodologies evaluated through the present study provided promising results, compared to HC2. Overall, CLART may be considered more useful for epidemiological studies, while NucliSENS® EasyQ® HPV test can be more appropriate to triage of HPV-positive women to potentially discriminate clinically relevant infections. The remaining evaluated tests (HC2, Cobas® 4800 HPV, Abbott RealTi*m*e HR HPV, APTIMA® HPV) are more suited for primary cervical cancer screening contexts due to their high sensitivity. In particular, Cobas® 4800 HPV and Abbott RealTi*m*e HR HPV, because of additional automation characteristics, should be useful for large screening studies. Also, the higher specificity of NucliSENS® EasyQ® HPV might avoid the discomfort and clinical consequences of colposcopy and invasive treatments.

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CHAPTER 6

Ana Oliveira, Nuno Verdasca, Ângela Pista

Contributions of the Authors:

Ana Oliveira was responsible for the research, writing and organization of this paper. She was also responsible for all the data analysis, including figures and tables, as well as laboratory work, namely DNA extraction and NucliSENS® EasyQ® HPV mRNA testing.

Nuno Verdasca contributed with proofreading and image rearrangements. He also conducted laboratory work, including DNA extraction and HPV genotyping.

Ângela Pista contributed with proofreading and scientific guidance.

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Use of the NucliSENS EasyQ HPV Assay in the Management of Cervical Intraepithelial Neoplasia

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Persistent infection by high-risk human papillomavirus is a necessary cause for cervical cancer. DNA-based human papillomavirus (HPV) assays show high sensitivity but poor specificity in detecting high-grade cervical lesions. Assays detecting mRNA of the oncoproteins E6 and E7 show higher specificity but lack either detection of all high-risk genotypes or the ability to specify the detected genotypes. The aim of this study was to evaluate the clinical performance of the NucliSENS EasyQ HPV assay in comparison with the Hybrid Capture 2 test (HC2) and the CLART Human Papillomavirus 2 assay (CLART), using a clinical cut-off of cervical intraepithelial neoplasia grade 2 or worse. In the 554 studied women, the lowest HPV positivity rate was detected for NucliSENS EasyQ HPV assay (55.1%), while HC2 and CLART showed similar results (HC2: 77.4%; CLART: 78.0%). In comparison with the other tests, the NucliSENS EasyQ HPV assay showed a lower clinical sensitivity (79.3% vs. 96.4% for HC2 and 95.9% for CLART) but a higher clinical specificity (72.6% vs. 42.8% for HC2 and 42.5% for CLART). Detection of E6/E7 mRNA transcripts may provide a higher specificity for cervical intraepithelial neoplasia grade 2 lesions or worse, since the oncogenic potential of HPV infection depends on the over-expression of these two oncoproteins. ***J. Med. Virol. 85:1235–1241, 2013.***

KEY WORDS: HPV; E6/E7 transcripts; mRNA testing; cervical intraepithelial neoplasia

INTRODUCTION

Epidemiological studies have established that certain human papillomavirus (HPV) genotypes are related etiologically to cervical cancer development [Kjaer et al., 2002; Clifford et al., 2003; Munoz et al., 2003; Bulkmans et al., 2007; Smith et al., 2007]. More than 120 HPV types have been identified, of which approximately 40 can infect the mucosa of the genital tract. According to the oncogenic potential, these HPV types can be classified into high-risk (HR), associated with premalignant lesions and cervical cancer, and low-risk (LR), found mainly in benign lesions [Munoz et al., 2003; Bernard et al., 2010]. Most infections will clear spontaneously, but persistent infection with HR-HPV is a strong predictor of the development of high-grade cervical intraepithelial neoplasia and cervical cancer [Kjaer et al., 2002; Schiffman et al., 2007].

HPV screening cohort studies have shown that HPV DNA testing has a higher sensitivity and negative predictive value than cytological testing for the detection of cervical intraepithelial neoplasia grade 2 or worse, although it has slightly lower specificity [Bulkmans et al., 2007; Ronco et al., 2008; Jeantet et al., 2009; Ratnam et al., 2010; Sorbye et al., 2010]. The majority of HPV tests detects the presence of HPV DNA, but it is possible to detect E6/E7 mRNA transcripts and thereby the presence of oncogenic activity [Molden et al., 2005; Dockter et al., 2009; Tropé et al., 2009]. The NucliSENS EasyQ HPV assay (bioMérieux S.A., Marcy l'Etoile, France) is a type-specific E6/E7 mRNA based test for oncogenic types 16, 18, 31, 33, and 45, with both HPV detection and genotyping performed in the same reaction [Molden et al., 2005; Tropé et al., 2009]. These five genotypes account for approximately 82% of all cervical carcinomas worldwide [Munoz et al., 2003; Smith et al., 2007]. The up-regulated

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expression of E6/E7 oncogenes is necessary for the malignant transformation in HPV related cancers [Molden et al., 2005; Ratnam et al., 2010; Burger et al., 2011]. Therefore, detection of E6/E7 mRNA transcripts may provide a more specific test for the detection of high-grade lesions and invasive carcinoma than DNA testing [Lie et al., 2005; Molden et al., 2005; Keegan et al., 2009; Burger et al., 2011], being suitable for triage of women who have been diagnosed with HR-HPV infection for risk stratification on cervical cancer development [Dockter et al., 2009; Jeantet et al., 2009].

The aim of this study was to evaluate the performance of the NucliSENS EasyQ HPV assay in comparison with the Hybrid Capture 2 (HC2) test and the CLART Human Papillomavirus 2 assay (CLART), using a clinical cut-off of cervical intraepithelial neoplasia grade 2 or worse.

MATERIALS AND METHODS

Study Design

The study group comprised 554 archived cervical samples from sexually active women, attending at primary healthcare clinics of the National Health Service and gynaecological outpatient clinics, who were referred to the National Institute of Health for opportunistic screening and for evaluation of HPV-associated lesions. The samples were selected consecutively during 5 months providing a high proportion of women at risk for cervical cancer and a broad range of outcomes and a high disease rate, which would enable accurate evaluation of sensitivity and specificity in a relative small sample.

Cervical samples were collected in ThinPrep PreservCyt medium (CyticUK, Crawley, West Sussex, UK) during clinical examination for cytological analyses. The residual liquid-based samples were used for HPV testing. The final diagnosis of the women with abnormal cytology was made by histological evaluation on colposcopically directed biopsies. As suggested by Wentzensen et al. [2009], 259 women (46.8%) were considered to have cervical intraepithelial neoplasia grade 1 or less (regarded as controls), while 295 (53.2%) were diagnosed as cervical intraepithelial neoplasia grade 2 or worse (regarded as disease cases). Both diagnoses (cytological and histological) were confirmed by experienced pathologists. No patient was sampled solely for the purpose of this research study.

All samples were tested by both the NucliSENS EasyQ HPV and the CLART (Genomica, Madrid, Spain) assays. In 532 samples the HC2 test (Digene, Gaithersburg, MD) was also performed. Each HPV test was carried out independently of each other according to the manufacturer's instructions. Nucleic acids were isolated from 1 ml of cellular suspension by using the NucliSENS easyMAG (bioMérieux S.A., Marcy l'Etoile, France) system, according to the manufacturer's instructions. Elution was performed in a final volume of 55 μl and stored at -20°C until HPV testing.

NucliSENS EasyQ HPV

Nucleic acid sequence-based amplification (NASBA) is based on an isothermal amplification and detection of E6/E7 mRNA from high-risk HPV 16, 18, 31, 33, and 45 using different molecular beacon probes. To verify the integrity of RNA in the specimen, the test includes a probe directed against the human U1 small nuclear ribonucleoprotein-specific mRNA. The use of two fluorescent dyes 6-carboxy fluorescein (6-FAM) for the HPV types 16, 31 and 33, and 6-carboxy-X-rhodamine (6-ROX) for the U1A protein and HPV types 18 and 45, allows simultaneous duplex amplification.

Hybrid Capture 2

The HC2 test is a sandwich capture molecular hybridization assay that uses a signal amplification detection method based on chemiluminescence. Thirteen HR-HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) can be detected with this test. The resultant DNA:RNA hybrids are captured on a microplate, and the emitted light is measured in a luminometer as relative light units (RLU). Samples were considered as positive if the ratio RLU/cut-off was >1.0 (equivalent to 1.0 pg HPV DNA/ml). All the cut-offs between 1 and 2.5 were retested and all were >1.0 RLU/cut-off (data not shown).

CLART Human Papillomavirus 2

This method uses biotinylated primers that amplify a 450 bp fragment within the HPV L1 region. Co-amplification of an 892 bp region of the CFTR gene and a 1,202 bp fragment of a transformed plasmid provides a control to ensure DNA extraction adequacy and PCR efficiency. Amplicons are detected by hybridization in a low-density microarray containing triplicate DNA probes specific to 35 types (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89). Semi-quantitative results can be obtained in an automatic reader.

Statistical Analysis

The clinical performance of the HPV tests was assessed on the basis of histological diagnosis, with cervical intraepithelial neoplasia grade 2 or worse serving as the disease endpoint. The sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) for each assay were calculated using 2×2 contingency tables with 95% confidence intervals (95% CI) computed using exact binomial methods. All P-values were obtained using the Fisher's exact test or McNemar χ^2 for comparison of matched-pair samples. P-values <0.05 were considered statistically significant.

The Cohen's kappa (κ) statistics was used to measure agreement between the NucliSENS EasyQ HPV assay and the HPV DNA tests. The HC2 test was used since it has a defined clinically cut-off value, and the CLART assay was used to better understand the analytical performance of NucliSENS EasyQ HPV assay. For comparison purposes, and data harmonization, it was only considered as a CLART-positive result the presence of one of the 13 HR-HPV types also detected in HC2 test (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) (CLART-13). It was also considered the analyses using the detection of only 5 HR-HPV types (HPV 16, 18, 31, 33, and 45) from CLART (CLART-5) for further comparison with NUcliSENS EasyQ HPV. All analyses were conducted using the SPSS (Statistical Package for the Social Sciences; SPSS, Inc., Chicago, IL) version 16.0 software.

RESULTS

Study Subjects

A total of 554 cervical samples from women aged 18–73 years old (mean age: 34.6 ± 10.4 years; median age: 33 years old) were studied for clinical evaluation of NucliSENS EasyQ HPV assay. The proportion of women ≥30 years of age was 65.3% (392/554). According to cytological grade there were 96 cases of normal cytology, 140 cases of atypical squamous cells of undetermined significance, 114 cases of low-grade squamous intraepithelial lesions, 194 cases of high-grade squamous intraepithelial lesions, and 10 cases of invasive cervical cancer. The histological analysis identified a total of 295 cases with a diagnosis of cervical intraepithelial neoplasia grade 2 or worse (including 6 cases of high-grade lesions with no histology available), comprising 129 cases of cervical intraepithelial neoplasia grade 2, 146 cases of cervical intraepithelial neoplasia grade 3, and 14 of cervical cancer, which resulted in a prevalence of cervical disease cases of 53.2% (295/554) in the studied set of samples. There were 128 cases of cervical intraepithelial neoplasia grade 1, along with 131 women having a normal cytology with no biopsy or with a negative histology, for a total of 259 cases representing a diagnosis of cervical intraepithelial neoplasia grade 1 or less (regarded as controls).

HPV Testing

Results of the HPV assays and "Clinical Diagnosis" parameter, as suggested by Wentzensen et al. [2009], were shown in Table I. Overall, HR-HPV types were detected in 305 cases (55.1%) with NucliSENS EasyQ HPV, accounting for 47.4% (91/192) of the positive cases in women aged <30 years, and for 59.1% (214/362) in women aged ≥30 years (data not shown). The E6/E7 mRNA transcripts were detected with NucliSENS EasyQ HPV in 4.2% of the cases with normal cytology. Regarding the cytological referral, the proportion of mRNA-positive cases increased progressively with the grade of cervical lesions, rising from 49.1% (56/114) in low-grade lesions, 77.8% (151/194) in high-grade lesions to 100% (10/10) in cervical cancer cases. Considering histology results, the same increasing proportion of mRNA-positive positivity was observed with 13.0% (17/131) in normal histology cases, 42.2% (54/128) in cervical intraepithelial neoplasia grade 1 cases, 76.7% (99/129) in cervical intraepithelial neoplasia grade 2 cases, 78.8% (115/146) in cervical intraepithelial neoplasia grade 3 cases, and 100% (14/14) in cervical cancer cases. HR-HPV types were detected in 412 cases (77.4%) with HC2, in 432 cases (78.0%) with CLART-13, and in 422 cases (76.2%) with CLART-5.

Clinical Performance of NucliSENS EasyQ HPV

Considering the "Clinical Diagnosis" parameter for further analysis combining results of cytology and histology, there were 46.8% (259/554) of cases with lesions of cervical intraepithelial neoplasia grade 1 or less (controls) and 53.2% (295/554) of cases with lesions of cervical intraepithelial neoplasia grade 2 or worse (disease cases). The sensitivity, specificity, positive predictive value and negative predictive value of the different tests in patients with cervical intraepithelial neoplasia grade 2 or worse are reported in Table II.

TABLE I. Characteristics of the Studied Samples

	N (%)
Referral Pap smear (n = 554)	
Normal cytology	96 (17.3)
Atypical cytology	140 (25.3)
Low-grade lesions	114 (20.6)
High-grade lesions	194 (35.0)
Cancer	10 (1.8)
Histology (n = 548)	
Normal	131 (23.9)
Cervical intraepithelial neoplasia grade 1	128 (23.4)
Cervical intraepithelial neoplasia grade 2	129 (23.5)
Cervical intraepithelial neoplasia grade 3	146 (26.6)
Cancer	14 (2.6)
Not Done	6
Clinical diagnosis (n = 554)	
Cervical intraepithelial neoplasia grade 1 or less (controls)	259 (46.8)
Cervical intraepithelial neoplasia grade 2 or worse (disease cases)	295 (53.2)
NucliSENS EasyQ HPV (n = 554)	
Positive	305 (55.1)
Negative	249 (44.9)
HC2 (n = 532)	
Positive	412 (77.4)
Negative	120 (22.6)
Not done	22
CLART-13 HR-HPV (n = 554)	
Positive	432 (78.0)
Negative	122 (22.0)
CLART-5 HR-HPV (n = 554)	
Positive	422 (76.2)
Negative	132 (23.8)

TABLE II. Clinical Performance for ≥CIN2 Based on Pap Smear and Biopsy Diagnosis

	Clinical diagnosis		Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
	Controls[a]	Disease cases[b]				
All women						
NucliSENS EasyQ HPV[c]						
Negative	188	61	79.3 (75.7–82.6)	72.6 (68.4–76.3)	76.7 (73.2–79.9)	75.5 (71.2–79.4)
Positive	71	234				
HC2[d]						
Negative	110	10	96.4 (93.6–98.1)	42.8 (39.9–44.7)	64.3 (62.5–65.5)	91.7 (85.4–95.6)
Positive	147	265				
CLART-13 HR-HPV[c]						
Negative	110	12	95.9 (93.3–97.7)	42.5 (39.4–44.5)	65.5 (63.7–66.7)	90.2 (83.7–94.5)
Positive	149	283				
CLART-5 HR-HPV[c]						
Negative	127	16	94.6 (91.7–96.7)	49.0 (45.8–51.4)	67.9 (65.8–69.4)	88.8 (82.9–93.1)
Positive	132	279				
Women <30 years						
NucliSENS EasyQ HPV[e]						
Negative	78	23	72.0 (63.5–79.3)	70.9 (64.6–76.4)	64.8 (57.2–71.5)	77.2 (70.3–83.2)
Positive	32	59				
HC2[f]						
Negative	45	2	97.5 (91.4–99.6)	41.3 (36.8–42.8)	54.9 (51.5–56.5)	95.7 (85.3–99.3)
Positive	64	78				
CLART-13 HR-HPV[e]						
Negative	45	2	97.6 (91.6–99.6)	40.9 (36.4–42.4)	55.2 (51.8–56.3)	95.7 (85.3–99.3)
Positive	65	80				
CLART-5 HR-HPV[e]						
Negative	52	2	97.6 (91.5–99.6)	47.3 (42.8–48.8)	58.0 (54.4–59.2)	96.3 (87.1–99.4)
Positive	58	80				
Women ≥30 years						
NucliSENS EasyQ HPV[g]						
Negative	110	38	82.2 (78.1–85.7)	73.8 (68.0–78.9)	81.8 (77.7–85.3)	74.3 (68.5–79.4)
Positive	39	175				
HC2[h]						
Negative	65	8	95.9 (92.5–98.0)	43.9 (39.4–46.7)	69.3 (66.8–70.8)	89.0 (80.0–94.7)
Positive	83	187				
CLART-13 HR-HPV[g]						
Negative	65	10	95.3 (92.0–97.5)	43.6 (39.0–46.8)	70.7 (68.3–72.4)	86.7 (77.4–92.9)
Positive	84	203				
CLART-5 HR-HPV[g]						
Negative	75	14	93.4 (89.9–96.0)	50.3 (45.3–54.1)	72.9 (70.2–74.9)	84.3 (75.9–90.5)
Positive	74	199				

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.
[a]Cervical intraepithelial neoplasia grade 1 or less.
[b]Cervical intraepithelial neoplasia grade 2 or worse.
[c]554 samples.
[d]532 samples.
[e]192 samples.
[f]189 samples.
[g]362 samples.
[h]343 samples.

Overall, the specificity of NucliSENS EasyQ HPV assay is significantly higher than that of HC2 and CLART-13 or CLART-5 (72.6%; 95% CI: 68.4–76.3 vs. 42.8% and 42.5% or 49.0%, respectively; $P = 0.000$), with a corresponding decrease in sensitivity (79.3%; 95% CI: 75.7–82.6 vs. 96.4% for HC2, 95.9% for CLART-13 or 94.6% for CLART-5, $P = 0.000$).

The sensitivity and specificity of the NucliSENS EasyQ HPV assay for the detection of disease was analyzed further in women <30 versus ≥30 years of age (Table II). This assay showed similar levels of specificity (70.9% vs. 73.8%) in women from both age groups, but it was identified an increase in sensitivity in women ≥30 years (82.2%) than in women <30 years (72.0%). For the DNA-based tests, the values were comparable in all age groups.

Overall, the concordance and agreement values between NucliSENS EasyQ HPV and the DNA-based tests were very similar (Table III).

The concordance between the NucliSENS EasyQ HPV and HC2 was 77.6%, with a kappa coefficient of 0.526, indicating fair agreement. Moreover, the positive agreement was 71.1%, and the negative agreement was 100%. The concordance between the NucliSENS EsayQ HPV and CLART-13 was 77.1%, with a kappa coefficient of 0.514. When the analysis

TABLE III. Analytical Performance of NucliSENS EasyQ HPV Versus CLART and HC2

	NucliSENS EasyQ HPV			
	Negative	Positive	Concordance % ± SD	Kappa (κ) (95% CI)
HC2[a]				
Negative	120	0	77.6 ± 1.6	0.526 (0.491–0.526)
Positive	119	293		
CLART-13 HR-HPV[b]				
Negative	122	0	77.1 ± 1.6	0.514 (0.480–0.514)
Positive	127	305		
CLART-5 HR-HPV[b]				
Negative	143	0	80.9 ± 1.6	0.598 (0.564–0.598)
Positive	106	305		

κ, kappa coefficient; CI, confidence interval; SD, standard deviation.
[a]n = 532 samples.
[b]n = 554 samples.

was performed regarding only the five types detected by NucliSENS EasyQ HPV, the concordance increased to 80.9%, with a kappa coefficient of 0.598.

DISCUSSION

Recent studies have shown that detection of HR-HPV, in particular of HPV 16 and 18, can be an important aspect to consider in cervical cancer screening programs and algorithms. Since most HR-HPV infections can clear spontaneously, a clinically useful HPV test needs to have an optimal clinical sensitivity and specificity in order to effectively detect cervical intraepithelial neoplasia grade 2 or worse [Cuzick et al., 2008; Meijer et al., 2009]. In this study, the performance of a commercially available HPV mRNA assay (NucliSENS EasyQ HPV) that allows the detection of oncogene transcripts for five HR-HPV types (HPV 16, 18, 31, 33, and 45) was compared with the HC2 test and the CLART genotyping assay. The HC2 test was used since it has a defined clinically cut-off value, and the CLART assay was used to better understand the analytical performance of NucliSENS EasyQ HPV assay. The clinical performance of this HPV mRNA assay was evaluated on a set of 554 cervical samples with cytological and/or histological results.

Tests with a better analytical sensitivity than that of HC2 will detect a large number of latent infections that are clinically irrelevant and lead to an overtreatment of women with transient HPV infections [Dockter et al., 2009; Jeantet et al., 2009; Meijer et al., 2009]. However, due to higher clinical specificity, RNA assays would be expected to detect fewer cervical intraepithelial neoplasia grade 2 cases than DNA assays since some of these lesions may be transient and could regress. In fact, mRNA testing applied to HPV screening programs may be a useful tool for the identification of clinically relevant HPV disease, being important in risk stratification of women with abnormal cytology and/or clinical symptoms [Jeantet et al., 2009]. Considering the higher specificity derived from the mRNA testing, NucliSENS EasyQ HPV was designed to detect E6/E7 oncogenic activity from the five HPV types most associated with cervical cancer worldwide [Clifford et al., 2003; Smith et al., 2007]. The identification of over-expression of these mRNAs in the presence of relevant clinical symptoms has pointed to a strong predictive value for HPV-associated cervical disease development and may, therefore, improve patient management [Jeantet et al., 2009; Sorbye et al., 2010].

This study evaluated the clinical performance of the NucliSENS EasyQ HPV assay in comparison with HC2 and CLART tests. As expected, the overall HR-HPV positivity rate was lower with NucliSENS EasyQ HPV (55.1%), when compared to HC2 (77.4%) and CLART-13 (78.0%) or CLART-5 (76.2%). In addition, the mRNA positivity rate increased with the severity of cytological grade, as shown in other studies [Lie et al., 2005; Tropé et al., 2009; Benevolo et al., 2011].

Both the HC2 and the CLART tests showed an identical clinical performance, with a clinical sensitivity around 96%, as published previously [Pista et al., 2011]. Overall, for NucliSENS EasyQ HPV assay, the clinical sensitivity was lower (79.3%), but with a corresponding increase in the clinical specificity (72.6%). In fact, the mRNA assay identified significantly fewer HPV-positive samples than HPV DNA tests, showing a much higher specificity in detecting clinically relevant disease. Similar data was reported by other studies [Szarewski et al., 2008; Keegan et al., 2009; Halfon et al., 2010; Ratnam et al., 2010]. However, this higher specificity attributed to mRNA HPV testing may not be due to the fact that NucliSENS EasyQ HPV only detects 5 HR-HPV types, as it was shown by the 49.0% of specificity for CLART-5.

Considering confirmed histologically cervical intraepithelial grade 2 or worse cases as the disease endpoint, the higher specificity of the NucliSENS EasyQ HPV assay will allow the reduction of false

positive results derived from DNA-based tests in transient HR-HPV infections, resulting in improved patient management and lower costs to the health care system. When used as a triage test, this assay can reduce the colposcopy referral in both abnormal cytology and low-grade lesions. On the other hand, using mRNA HPV testing in DNA-positive women will allow risk stratification reducing colposcopy referral more than cytology [Szarewski et al., 2008; Dockter et al., 2009; Jeantet et al., 2009; Sorbye et al., 2010; Benevolo et al., 2011].

This study demonstrated a concordance between NucliSENS EasyQ HPV and HC2 or CLART tests over than 77%, despite the difference in the number of oncogenic HPV types detected by all assays. The five oncogenic HPV types detected by NucliSENS EasyQ HPV were found in 79.3% of cervical intraepithelial neoplasia grade 2 or worse, which is in concordance with high-grade cervical lesions prevalence rate studies [Clifford et al., 2003; Smith et al., 2007]. There were 61 (20.7%) cases of cervical intraepithelial neoplasia grade 2 or worse which were negative by NucliSENS EasyQ HPV. Of these, 17 (27.9%) were also negative for HPV DNA or were infections of other HR-HPV types not detected by the mRNA assay.

This study has some limitations. First, it is a cross-sectional study, precluding the determination of the test's longitudinal positive or negative predictive value. On the other hand, NucliSENS EasyQ HPV assay lack the detection and typing of all HR-HPV genotypes.

In conclusion, the higher specificity for cervical intraepithelial neoplasia grade 2 or worse provided by the NucliSENS EasyQ HPV assay may be useful in the identification of the majority of women with lesions that could progress, since the oncogenic potential of HPV infection depends on expression of E6/E7 oncoproteins. This assay may have a higher triage effect as a reflex test in HPV DNA-positive women and may be more cost effective, reducing the number of positive cases that are referred to colposcopy.

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CHAPTER 7

Ana Gradíssimo, Nuno Verdasca

Contributions of the Authors

Ana Gradíssimo was responsible for the research, writing and organization of this paper. She was also responsible for all the data analysis, including figures and tables, as well as laboratory work, namely DNA extraction, HPV genotyping and real-time PCR amplification.

Nuno Verdasca contributed with proofreading and image rearrangements. He also conducted laboratory work, including DNA extraction and HPV genotyping.

ORIGINAL ARTICLE

Viral Load of HPV 16 and 18 as Biomarker for Cervical Disease

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Running Title: Viral load of HPV 16 and 18.

ABSTRACT

Infection with high-risk human papillomavirus (HPV) is considered to play a central role in cervical carcinogenesis. Molecular markers may increase HPV detection specificity and predict the risk of disease progression. Viral load has been proposed as a marker for progression to cervical precancerous lesions but it has been confirmed only to HPV 16. The aim of this study was to evaluate the clinical significance of the viral load of HPV 16 and 18. Cervical smears from 499 HPV 16 and/or 18-positive women (117 with normal cytology, 84 with ASC-US, 134 with LSIL, 149 with HSIL, and 15 with ICC) were evaluated. Viral load was determined through real-time PCR by quantitation of the E6 gene for HPV 16 and 18, while the albumin gene was used as a housekeeping gene to estimate the number of human cells. HPV 16 and 18 viral load increased with severity of cervical abnormality ($P < 0.001$ and $P < 0.001$, respectively). Regarding HPV 18 cases, mean viral load increased highly in LSIL cases, when compared to normal cytologies, but decreased in high-grade lesions; this finding might be related to the predisposition of this genotype to infect glandular cells, which are less detectable through cytology. These results show that viral load quantitation for HPV 16 and 18 may constitute an important biomarker in predicting cervical cancer development, and in improving patient management among women infected with these oncogenic HPV genotypes.

Keywords: Cervical cancer, human papillomavirus, molecular marker, viral load, risk assessment.

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INTRODUCTION

Worldwide, cervical cancer is the second most common cancer among women, and virtually all cervical cancers are related to human papillomavirus (HPV) infection [1,2]. HPV 16 and 18 genotypes are the most frequently related to cervical cancer and are responsible for more than 70.0% of the cases [3-5]. HPV 18 is more frequently detected in adenocarcinoma and adenosquamous carcinoma, while HPV 16 is more often associated to squamous cell carcinoma [6,7].

It has been well established that persistent high-risk HPV infection is the major risk factor for cervical cancer [8-11]. The average time from HPV infection to development of cervical cancer is 10 to 15 years, and the likelihood of progression from infection to disease increases with age [2]. In Portugal, cervical cancer incidence rate has been estimated in 12.2/100,000 and the mortality rate in 3.6/100,000 [12]. HPV 16 is the most frequently detected genotype among Portuguese women independently of the cytological grade (19.7%), while for HPV 18 a much lower prevalence was determined (4.4%) [13].

Several potential markers of premalignant lesions development and cervical cancer have been evaluated, among which HPV viral load has been described as a prognostic marker for cervical intraepithelial neoplasia (CIN) development [14-17]. Several studies have shown that high HPV DNA load is associated with persistence of HPV infection and with an increased risk for the development of cervical intraepithelial neoplasia grade 2 or worse (CIN2+) and invasive cervical carcinoma (ICC) [16-21]. Therefore, viral load assessment together with HPV 16/18 testing may help to identify women at risk of CIN2+ or ICC development [22]. However, the clinical value of viral load is not consistent across studies, which may reflect, in part, the use of different quantitation assays [23,24].

Since HPV DNA testing has become available, and has demonstrated high sensitivity and reproducibility, it was recommended that HPV DNA tests could be integrated in cervical cancer screening programs and in the management of women at risk of premalignant lesions development [10]. However, it is still controversial if the primary screening test should be the cytology or the HPV DNA test [16,17,25]. In this perspective, it is essential to perform an adequate triage[5] of HPV-positive women. Determining the clinical significance of potential biomarkers (which are more specific) may be useful to a further risk assessment evaluation and improved management of HPV-infected women. The aim of this study was to determine the predictive value of HPV viral load quantitation for cervical cancer development, and assess its clinical prognostic significance among Portuguese women infected by HPV 16 and 18 genotypes.

[5] Term applied to selection of women with abnormal cytology using HPV DNA test for better risk evaluation in a screening program.

MATERIALS AND METHODS

Biological Samples

Frozen DNA aliquots (n = 499) from cervical samples that had been previously genotyped by CLART® Papillomavirus Humano 2 (Genomica, Madrid, Spain) were selected for the present study, comprising 361 cases positive for HPV 16, 138 cases positive for HPV 18, among which 17 were positive for both genotypes. This subset group was selected from an enlarged prospective study, as previously described (Chapter 3). The samples were stratified according to host characteristics into seven age groups (18-19; 20-24; 25-29; 30-39; 40-49; 50-59; 60-65), and in five cytological categories (NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial neoplasia; HSIL, high-grade intraepithelial neoplasia; and ICC, invasive cervical carcinoma), which corresponded to five histological categories (normal, negative histology; CIN1-3, cervical intraepithelial neoplasia grade 1 to 3; and ICC, invasive cervical carcinoma). Cytological and histological diagnoses had been previously confirmed by experienced pathologists and made available to the Human Papillomavirus Reference Laboratory at the National Institute of Health (INSA) for opportunistic screening and HPV testing (Chapter 3). Therefore, no patient was sampled solely for the purpose of this research study. Women or clinician were not contacted with any result, other than HPV testing, nor would it be used to influence their management.

Real-time quantitative PCR

HPV 16 and 18 viral loads were assessed by real-time PCR amplification of the E6 gene, using the human housekeeping albumin gene as a reference for the estimation of the number of human host cells, as previously described [26]. Briefly, real-time quantitative PCR was carried out in a 96-well reaction 0.2 ml microplate on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For each sample, absolute quantitation of E6 and albumin genes was performed in a duplex real-time PCR reaction, where each sample was tested in duplicate simultaneously in the same plate. Commercially available standards with the complete viral genomes cloned into plasmids (*HPV 16 Genome for Real Time Standards* and *HPV 18 Genome for Real Time Standards*; CLONIT, Milano, Italy) were diluted in sterile water from 200,000 copies in a 2-fold 8-dilution series. Standards were carried out in duplicate in every plate, as well as a negative control. The reaction was performed in a 25 µl mixture containing TaqMan® Gene Expression Master Mix (Invitrogen Corporation, Carlsbad, CA, USA), 400nM of forward and reverse primers for both E6 and albumin genes, and 2 µl of each DNA sample was added to the reaction mixture. Amplification conditions were as follows: 2 min at 50ºC, 10 min at 95ºC, a two-step cycle at 95ºC for 10 secs, and 60ºC for 1 min, for a total of 45 cycles.

Viral load is expressed as the number of HPV 16 and 18 copies per million of normal human cells according to the following formula:

$$\text{Viral Load } \left(\frac{\text{HPV copies}}{10^6 \text{ cells}}\right) = \left(\frac{\text{Number of HPV copies}}{\frac{\text{Number of albumin copies}}{2}}\right) \times 10^6$$

Statistical Methods

The histological result was applied as the gold-standard in assessing the predictive value of the viral load in cervical cancer development using the cytological result as baseline. Multiple infections with HPV 16 and 18 were counted has duplicates. Quantitative results from the real-time PCR amplification were log transformed for correlation analyses and for graphical display. Mann-Whitney tests were used to determine differences in viral load between samples of normal cytology (reference group) and samples with CIN (test group). The Kruskal-Wallis non-parametric methods were used to discriminate differences in HPV viral load among the different cytological and histological categories. A two-sided P value ≤ 0.05 was considered statistically significant. All analyses were performed by using the IBM SPSS Statistics version 20.0 (IBM Corporation Inc., Armonk, NY, USA).

RESULTS

Study Population

The samples selected for viral load determination were stratified according to age and clinical diagnosis (Table 1). Regarding the HPV 16-positive samples, the mean age ranged between 31.8 ± 9.6 and 47.2 ± 16.5 (mean age ± standard deviation (SD)), when comparing normal histology to squamous cell carcinoma cases, respectively. Regarding HPV 18-positive samples, the mean age ranged between 32.2 ± 11.1 and 43.2 ± 13.4, for normal histology and adenocarcinoma cases, respectively.

Viral Load Quantitation

The mean viral load stratified by age and cytology is presented in Table 2. Overall, the value of the mean viral load for HPV 16 was 2.7×10^8 ($\pm 3.6 \times 10^9$), among which 1.1×10^7 ($\pm 1.5 \times 10^7$) was determined in ICC cases. For HPV 18, the mean viral load value was 7.1×10^9 ($\pm 8.1 \times 10^{10}$), among which 3.8×10^9 ($\pm 9.9 \times 10^9$) was determined for ICC cases. Moreover, for HPV 16 there was a statistical significant association between viral load and older women (≥ 30 years) ($P < 0.001$), in opposition to HPV 18-positive cases where no statistical association was identified ($P = 0.221$) (Table 2).

Table 1: Characteristics of the studied samples

	Cases (%) (N=499)	HPV 16-positive (%) (n=361)	HPV 18-positive (%) (n=138)
Age (mean; SD)	32.9 ± 9.9†	32.6 ± 9.9	32.6 ± 8.3*
18-19	9 (1.9)	5 (1.4)	4 (3.3)
20-24	79 (16.4)	59 (16.3)	20 (16.4)
25-29	136 (28.2)	104 (28.8)	32 (26.2)
30-39	167 (34.6)	126 (34.9)	41 (33.6)
40-49	60 (12.4)	42 (11.6)	18 (14.8)
50-59	17 (3.5)	12 (3.3)	5 (4.1)
60-65	15 (3.1)	13 (3.6)	2 (1.6)
nd	16	0	16
Cytology			
NILM	117 (23.4)	78 (21.6)	39 (28.3)
ASC-US	84 (16.8)	53 (14.7)	31 (22.4)
LSIL	134 (26.9)	100 (27.0)	34 (24.6)
HSIL	149 (29.9)	122 (33.8)	27 (19.6)
ICC	15 (3.0)	8 (2.2)	7 (5.1)
Histology			
Normal	45 (9.0)	45 (12.5)	0 (0.0)
CIN1	143 (28.7)	90 (24.9)	53 (38.4)
CIN2	194 (38.9)	129 (35.7)	65 (47.1)
CIN3	109 (21.8)	93 (25.8)	16 (11.6)
ICC	8 (1.6)	4 (1.1)	4 (2.9)

NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma, including adenocarcinoma; CIN, cervical intraepithelial neoplasia grade 1 to 3; †, n=483; *, n=122; nd, not determined.

Viral load values for HPV 16 and 18 varied among the different clinical cytological or histological categories ($P < 0.001$). The mean viral load for HPV 16 was significantly higher in HSIL ($P < 0.001$), when compared to normal cytology. HPV 16 viral load increased with the severity of cervical lesion, but a decrease was observed in ICC cases. Considering the histological diagnosis, the HPV 16 mean viral load increased from negative histologies to CIN1 and CIN2 cases ($P = 0.029$), and then decreased slightly in CIN3 and ICC cases. From HPV 16-positive cases, 55.4% (200/361) corresponded to single infections, and the remaining 44.6% (161/361) were co-infections with HPV 16 and other high-risk genotypes, namely HPV 31 (15.5%; 25/161) and HPV 51 (13.0%; 21/161). However, the mean viral load of HPV 16 was not statistically associated to clinical diagnosis, whether it was single or multiple infections ($P = 0.174$) (Fig.1).

For HPV 18 viral load, significantly higher values were observed in LSIL ($P = 0.001$), when compared to normal cytology. However, HPV 18 high viral load was not associated with HSIL and ICC cases, as it was observed a slight decrease in the mean values ($P = 0.516$) (Table 2). Considering the histological diagnosis, the mean viral load for HPV 18 was consistent among CIN lesions, and then decreasing in ICC cases ($P = 0.112$) (Fig.1). From HPV 18-positive cases, 55.1% (76/138) corresponded to single infections, and the remaining 44.9% (62/138) were co-infections with HPV 18 and other high-risk genotypes, namely HPV 16 (27.4%; 17/62) and HPV 31 (17.7%; 11/62). Moreover, the mean viral load of HPV 18 was not associated with the clinical diagnosis for single

infections, when analyzed in comparison to HPV 18 multiple infections that were statistically significant (P = 0.003) (data not shown). Fig. 2 show logarithmic mean viral load values for HPV 16 and 18 by cytological/histological categories.

Table 2: Mean viral load for HPV 16 and 18 stratified by age and cytological diagnosis

	Overall*		≤ 30 years		> 30 years	
	HPV 16 (n = 361)	**HPV 18 (n = 138)**	**HPV 16 (n = 182)**	**HPV 18 (n = 60)**	**HPV 16 (n = 179)**	**HPV 18 (n = 62)**
Cytological Group						
NILM (n = 117)						
Number of cases	78	39	45	24	33	15
Mean viral load (± SD)	$2.3x10^6$ ($\pm 9.7x10^6$)	$5.5x10^5$ ($\pm 1.8x10^6$)	$6.4x10^5$ ($\pm 1.5x10^6$)	$5.7x10^7$ ($\pm 2.3x10^8$)	$2.7x10^8$ ($\pm 1.0x10^9$)	$2.8x10^4$ ($\pm 4.1x10^4$)
ASC-US (n = 84)						
Number of cases	53	31	32	13	21	18
Mean viral load (± SD)	$3.7x10^6$ ($\pm 1.1x10^7$)	$6.5x10^4$ ($\pm 1.1x10^5$)	$3.2x10^6$ ($\pm 1.0x10^7$)	$7.8x10^4$ ($\pm 1.4x10^5$)	$4.5x10^6$ ($\pm 1.2x10^7$)	$5.6x10^4$ ($\pm 8.2x10^4$)
LSIL (n = 134)						
Number of cases	100	34	63	17	37	17
Mean viral load (± SD)	$1.1x10^8$ ($\pm 6.6x10^8$)	$1.2x10^7$ ($\pm 4.4x10^7$)	$1.3x10^7$ ($\pm 3.4x10^7$)	$2.1x10^7$ ($\pm 6.0x10^7$)	$2.8x10^8$ ($\pm 1.1x10^9$)	$2.6x10^6$ ($\pm 4.1x10^6$)
HSIL (n = 149)						
Number of cases	122	27	40	6	82	8
Mean viral load (± SD)	$6.9x10^8$ ($\pm 6.3x10^9$)	$3.4x10^6$ ($\pm 7.6x10^6$)	$7.0x10^7$ ($\pm 2.1x10^8$)	$4.6x10^6$ ($\pm 3.9x10^6$)	$9.9x10^8$ ($\pm 7.7x10^9$)	$2.3x10^6$ ($\pm 2.9x10^6$)
ICC (n = 15)						
Number of cases	8	7	2	0	6	4
Mean viral load (± SD)	$1.1x10^7$ ($\pm 1.5x10^7$)	$1.5x10^6$ ($\pm 3.7x10^6$)	$3.5x10^7$ ($\pm 5.4x10^7$)	-	$3.1x10^6$ ($\pm 3.5x10^6$)	$3.0x10^6$ ($\pm 5.2x10^6$)
Histological Group						
Normal (n = 45)						
Number of cases	45	0	31	0	14	0
Mean viral load (± SD)	$1.5x10^5$ ($\pm 3.2x10^5$)	-	$1.3x10^5$ ($\pm 3.4x10^5$)	-	$1.7x10^5$ ($\pm 2.9x10^5$)	-
CIN1 (n = 143)						
Number of cases	90	53	48	28	42	25
Mean viral load (± SD)	$1.3x10^8$ ($\pm 6.6x10^8$)	$8.4x10^6$ ($\pm 3.7x10^7$)	$2.5x10^7$ ($\pm 1.2x10^8$)	$6.5x10^7$ ($\pm 2.2x10^8$)	$2.6x10^8$ ($\pm 9.6x10^8$)	$5.2x10^5$ ($\pm 2.3x10^6$)
CIN2 (n = 193)						
Number of cases	128	65	62	29	66	30
Mean viral load (± SD)	$1.3x10^8$ ($\pm 6.5x10^8$)	$3.8x10^6$ ($\pm 1.4x10^7$)	$2.7x10^7$ ($\pm 1.1x10^8$)	$6.4x10^6$ ($\pm 2.1x10^7$)	$2.3x10^8$ ($\pm 8.7x10^8$)	$1.2x10^6$ ($\pm 2.7x10^6$)
CIN3 (n = 110)						
Number of cases	94	16	41	3	53	6
Mean viral load (± SD)	$4.8x10^7$ ($\pm 1.7x10^8$)	$2.8x10^6$ ($\pm 3.6x10^6$)	$2.9x10^7$ ($\pm 1.2x10^8$)	$5.6x10^6$ ($\pm 5.1x10^6$)	$6.1x10^7$ ($\pm 1.9x10^8$)	$3.1x10^6$ ($\pm 3.8x10^6$)
ICC (n = 8)						
Number of cases	4	4	0	0	4	1
Mean viral load (± SD)	$3.8x10^6$ ($\pm 4.4x10^6$)	$3.4x10^4$ ($\pm 3.3x10^4$)	-	-	$3.8x10^6$ ($\pm 4.4x10^6$)	$2.6x10^{10}$ (-)

NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma, including adenocarcinoma; CIN1-3, cervical intraepithelial neoplasia grade 1 to 3; SD, standard deviation; *, Overall mean viral load included 16 samples without age information.

Multiple infections simultaneously harboring HPV 16 and 18 were identified in 17 samples (3 NILM, 2 ASC-US, 7 LSIL, and 5 HSIL). Overall, the viral load was higher for HPV 16, when compared to HPV 18 in these multiple infections, with no statistical significance (P = 0.163).

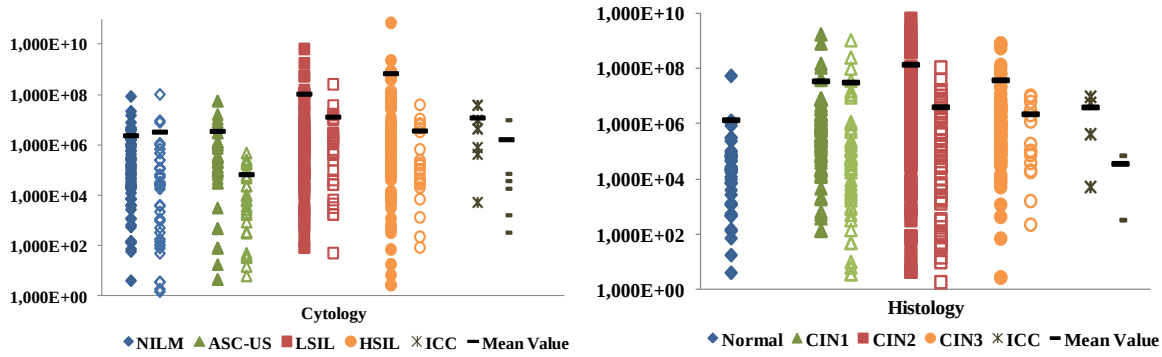

Fig. 1: Distribution of HPV 16 and 18 viral load by cytological and histological grade. NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma, including adenocarcinoma; CIN, cervical intraepithelial neoplasia grade 1 to 3. Filled symbols correspond to HPV 16 cases and empty symbols correspond to HPV 18 cases.

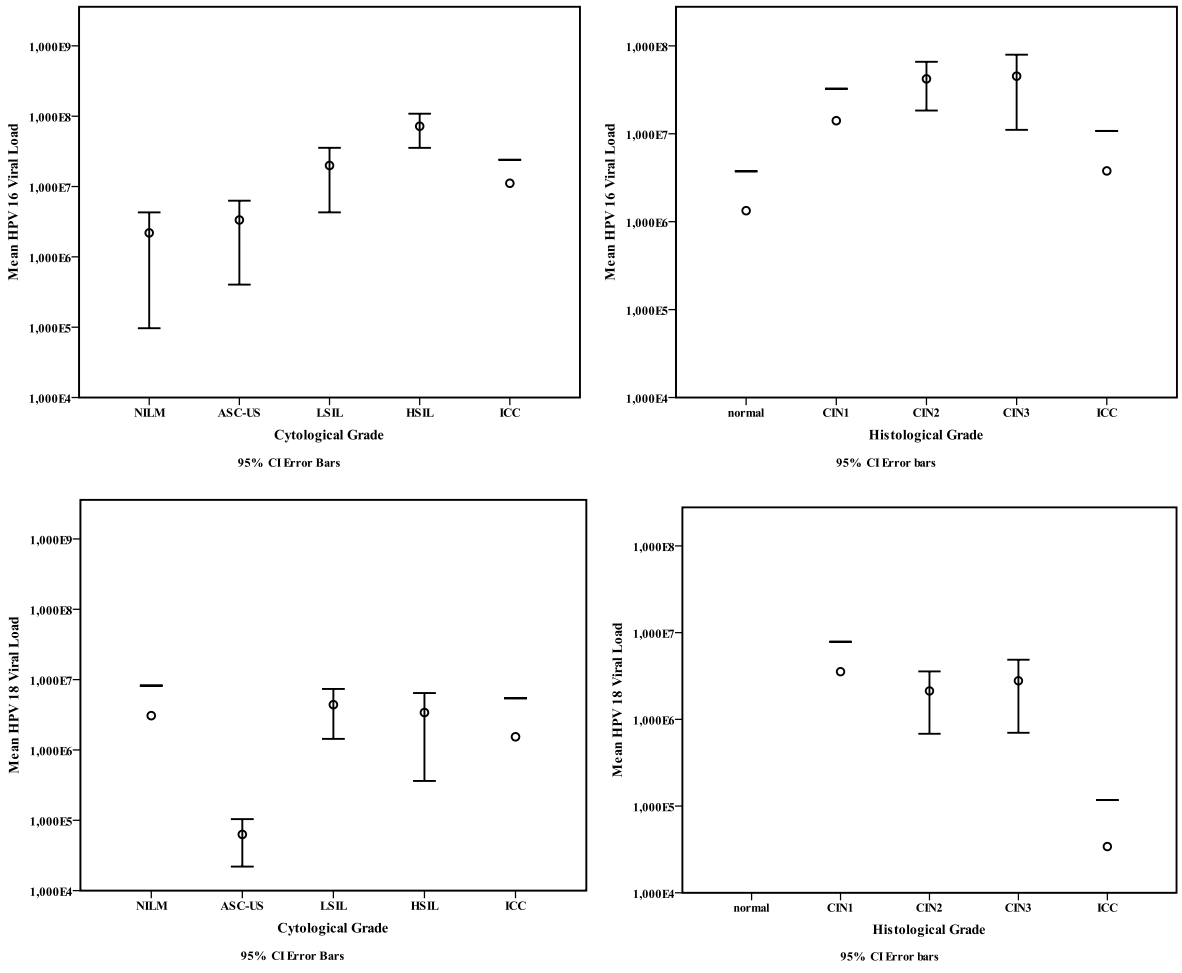

Fig. 2: Comparison of HPV 16 (up) and HPV 18 (bottom) viral load according to clinical diagnosis. Data is presented as T-plots, representing the 5th and 95th percentiles. NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma; CIN, cervical intraepithelial neoplasia grade 1 to 3.

DISCUSSION

Viral persistence is required for cervical cancer carcinogenesis, and increased risk has been associated with early high viral loads [27,28]. Studies that evidenced an increase in viral load measurements during HPV infection have shown an association with a modest increased risk of developing cytological abnormalities [29-33]. HPV 16 is the only genotype for which viral load may predict viral persistence and progression to cervical cancer [16,21,34]. The aim of this study was to identify the clinical significance of HPV 16 and 18 viral loads as a potential biomarker for cervical cancer development, by applying a real-time quantitative PCR approach that constitutes the gold standard for HPV viral load quantitation.

Although the significance of HPV viral load is specific of the infecting genotype, the quantitation of HPV load may clarify between clearance and persistence regarding HPV-infected women. Several methods have been described for HPV quantitation, all considering a real-time quantitative PCR approach [35-40]. In fact, the advances in molecular biology technologies not only exposed the importance of HPV viral load quantitation but also revealed appropriate for the determination of the prognostic significance of several viral genes, so that these methodologies have been tested for HPV epidemiological and clinical purposes. However, real-time techniques may be quite expensive especially beacuse they are based on TaqMan® oligoprobes (Invitrogen Corporation, Carlsbad, CA, USA) and limited/specific for each HPV genotype [40]. The importance of this biomarker in cervical carcinogenesis has prompted many evaluations but the prognostic value is still under investigation. In this assessment, the TaqMan® technology was used due to its high specificity, and because it enables the possibility of duplex real-time quantitation. While many authors established an association between HPV viral load and the cytological diagnosis [28,38,41-46], in this study the histological result was applied as the gold-standard to assess the clinical significance of HPV viral load in cervical disease. It was intended to understand if high viral loads were more frequent among high-grade cervical lesions (CIN2 or worse), and, if so, to evaluate the applicability of this methodology to clinical settings while reducing colposcopy and biopsy referral. Recurring only to the cytological sample one can predict the risk of persistent infection and cervical lesions development, while eliminating the need for unnecessary invasive procedures that induce anxiety and morbidity to HPV-infected women.

An association between HPV 16 viral load and cervical cancer development has been established in this study. However, it is hard to determine whether such an association could be extended to less carcinogenic and less prevalent genotypes [47]. The present study evidenced an association between the severity of cervical lesions and HPV 16 and 18 viral loads, which is in agreement with other studies [26,48,49]. Regarding HPV 16-positive cases, the mean viral load increased significantly with the severity of cervical lesion. In fact, considering that the higher values

were observed among high-grade cervical lesions, viral load quantitation could be considered an important molecular marker for the clinical evaluation of HPV 16 infections. It was only observed a decreased amount of HPV 16 viral load among ICC cases, suggesting that after invasion and cancer development, viral load may be no longer necessary to maintain cervical cancer progression. HPV 16 viral load was significantly higher among LSIL cases, when compared to ASC-US cases, suggesting an increased risk for persistence, which is a recognized risk factor for cervical cancer development.

For HPV 18, it was observed an increase in the mean viral load values from normal cytologies to LSIL lesions, and then a decrease among HSIL and ICC cases. This could suggest that the higher viral loads determined in low-grade cervical lesions may favor the persistence of HPV infection and its progression to high-grade cervical lesions and cervical cancer. However, considering that among HSIL and ICC cases the mean HPV 18 viral load decreased, one could consider that during the final stages of carcinogenesis, viral load might not be the major factor related to cervical cancer development. In fact, the decrease in viral load values among high-grade lesions has been previously proposed [47,50,51]. The predisposition of HPV 18 for infecting glandular cells and its detection in adenocarcinomas that are more difficult to detect cytologically could also explain these findings. Moreover, the age range from normal histology to adenocarcinoma was 11 years, suggesting a more rapid progression to cervical cancer, when compared to HPV 16-positive cases (15.4 years) [6].

It was proposed by Saunier *et al.* [52] a cut-off value of 22,000 copies/10^3 cells for HPV 16 viral load, in which values higher than the cut-off corresponded to an associated risk for cervical lesion development. In the present study, using this cut-off, 46 HPV 16-positive cases (12.7%) (1 normal, 9 CIN1, 25 CIN2, and 11 CIN3), and eight (5.8%) of HPV 18-positive cases (5 CIN1, 2 CIN2, and 1 ICC), could be considered at risk. Assuming that a cervical lesion develops following infection by one specific genotype (one virus-one lesion concept), genotype-specific quantitation could be required to determine the true clinical significance of HPV 16 and 18 viral loads as it was performed in this study [53,54].

Multiple HPV infections have been considered as a risk factor for the development of high-grade cervical lesions [55,56]. In the present study, a high proportion of women infected with HPV 16 and 18 were also co-infected with other high-risk HPV genotypes, namely HPV 31, 33 and 51. However, differences in HPV 16 and 18 viral loads were not observed between women co-infected or not co-infected with other high-risk HPV genotypes. The methodology used in this study is specific of each genotype, only quantifying HPV 16 or 18, with statistical significant association between multiple infections and HPV 18 viral load. It is of note that among cases with co-infection by HPV 16+18, the HPV 16 viral load was constantly higher than the quantified for HPV 18 in the same sample.

The results of the present study suggest that the determination of a high viral load during the early stages of HPV 16 and 18 infections may be an important risk predictor of malignant

progression, and women with high HPV 16 and 18 viral loads should be closely monitored because of their major aggressive potential. In conclusion, viral load quantitation constitutes a valuable biomarker for predicting the risk of cervical cancer development, improving patient management.

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CHAPTER 8

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Contributions of the Authors:

Ana Gradíssimo was responsible for the research, writing and organization of this paper. She was also responsible for the data analysis, including figures and tables, as well as laboratory work, namely DNA extraction, HPV genotyping and real-time PCR amplification.

Cândida Delgado was responsible for the implementation of this methodology. She also contributed with proofreading and data analysis.

Nuno Verdasca contributed with proofreading and image rearrangements. He also conducted laboratory work, including DNA extraction and HPV genotyping.

Ângela Pista contributed with proofreading and scientific guidance.

*, contributed equally to this work.

ORIGINAL ARTICLE

Prognostic value of human papillomavirus types 16 and 18 DNA physical status in cervical intraepithelial neoplasia

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Running Title: Physical status of HPV 16 and 18.

ABSTRACT

Human papillomavirus (HPV) integration into the host genome is a critical event for cervical carcinogenesis. The aim of this study was to assess the value of the physical status of HPV 16 and 18 DNA as a disease marker for cervical cancer development. A subset of 499 DNA samples previously genotyped as HPV 16 or 18 was selected, comprehending 45 (9.0%) samples from normal histology cases, while 143 (28.7%) were from CIN1, 194 (38.9%) from CIN2, 109 (21.8%) from CIN3 and eight (1.6%) from ICC. The physical status of both HPV genotypes was assessed by calculating the E2/E6 ratio through real-time quantitative PCR. Among HPV 16 infections, 19.7% (71/361) were found in the episomal form, 74.0% (267/361) presented concomitant forms, and 6.4% (23/361) were in the linear form. Regarding HPV 18 infections, 13.0% (18/138) were episomal, 55.8% (77/138) presented concomitant forms, and 31.2% (43/138) were in the linear state. There was a statistically significant association between HPV 16 and 18 DNA integration status and disease state ($P < 0.001$). Furthermore, especially for HPV 18, the determination of the DNA physical status may become a useful biomarker in predicting cervical cancer risk development, where a lower E2/E6 ratio would be more frequent among precancerous lesions.

Keywords: Cervical cancer, human papillomavirus 18, molecular marker, physical status, risk assessment.

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INTRODUCTION

Infection by human papillomavirus (HPV) is aetiologically linked to cervical cancer development [1,2]. HPV 16 and 18 genotypes are the most carcinogenic, with a higher prevalence in cervical cancer cases worldwide [3-5]. Portugal has one of the highest incidence rates registered in Europe (12.2/100,000), as well as a high mortality rate (3.6/100,000) [6]. HPV 16 is the most frequently detected genotype independently of the cytological grade (19.7%), while HPV 18 has a low prevalence in Portuguese women (4.4%) [7].

Most HPV-related cervical intraepithelial precursor lesions are known to regress spontaneously, which implies that although HPV is a necessary cause for cervical cancer development, additional factors must play a role in the progression of those lesions. Many studies have focused on the identification of virological markers that could be predictive of the progression from cervical intraepithelial lesions to cervical cancer, but also act as an adjunct molecular approach to identify HPV-infected women at risk for cervical cancer development [8-11].

Integration of the viral DNA into the host cell genome could constitute a virological marker, as it is often considered as a pre-requisite for the development of malignant lesions. On the other hand, the physical status of HPV DNA (episomal, linear or concomitant/mixed forms) has been considered as a marker of disease progression [12-14], and the absolute quantitation of E2 and E6 genes by real-time PCR, followed by the calculation of the E2/E6 ratio has been proposed for the identification of the different forms of the viral DNA [15].

The HPV viral integration into the host genome results from a viral genome disruption, during which occurs a partial loss of the E2 gene, promoting the linearization and integration of the viral genome, which will induce deregulated production of E6 and E7 oncoproteins [16,17]. Therefore, the discrimination of the integrated forms in HPV-positive cases could offer a more specific target of disease risk. In addition, recent data suggest that integration frequency in pre- and malignant lesions varies according to HPV genotype, where the estimates of integration vary from 5.0% to 100.0% in high-grade cervical lesions (HSIL) to no evidence of integration, namely among low-grade cervical lesions (LSIL) [18-20].

The aim of this study was to investigate the level of integration of HPV 18 and its association with progression from cervical intraepithelial neoplasia (CIN) to the development of invasive cervical cancer (ICC), in comparison with HPV 16. The value of the determination of physical status for both HPV 16 and 18 genotypes, as a virological marker for cervical cancer development will also be evaluated.

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MATERIALS AND METHODS

Biological Samples

Frozen DNA aliquots (n = 499) obtained from cervical samples, previously genotyped as HPV 16 (n = 361) and HPV 18 (n = 138) were selected for HPV integration evaluation from an enlarged prospective study (Chapter 3). Data concerning the cytological and histological evaluations, as well as age from HPV-infected women were made available for the purposes of this study. As previously described (Chapter 7), the age of HPV 16-infected women varied from 18 to 65 years (mean age 32.6 ± 9.9 years; median 30 years). Histological grade comprehended 12.5% (45/361) with negative histology, 24.9% (90/361) with CIN1, 35.7% (129/361) with CIN2, 25.8% (93/361) with CIN3 and 1.1% (4/361) with ICC. Age of HPV 18-infected women varied from 18 to 64 years (mean age 32.6 ± 8.3 years; median 31 years). All these women presented histological abnormalities: 38.4% (53/138) CIN1, 47.1% (65/138) CIN2, 11.6% (16/138) CIN3, and 2.9% (4/138) adenocarcinomas (Chapter 7).

HPV 16 and 18 DNA physical status assessment

The physical status of HPV 16 and 18 DNA was assessed by real-time PCR amplification of E2 and E6 genes, as previously described [21]. Real-time quantitative PCR was carried out in a 96-well reaction plate on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For each sample, absolute quantitation of E2 and E6 genes was performed in duplicate, and simultaneously in the same plate. Commercially available standards with the complete viral genomes cloned into plasmids (*HPV 16 Genome for Real Time Standards* and *HPV 18 Genome for Real Time Standards*; CLONIT, Milano, Italy) were diluted in sterile water from 200,000 copies in a 2-fold 8-dilution series. Standards were carried out in duplicate in every plate, as well as a negative control. The reaction was performed in a 25 µl mixture containing 1x SYBR® GreenER™ qPCR Supermix for ABI PRISM® (Invitrogen Corporation, Carlsbad, CA, USA), 400nM of forward and reverse primers for E2 and E6 regions [22], and 5 µl of DNA from each sample was added to the reaction mixture. Amplification conditions were as follows: 10 min at 95ºC, a two-step cycle at 95ºC for 10 secs, and 60ºC for 1 min, for a total of 40 cycles. HPV 16 and 18 DNA physical status was assessed following the ratio calculation determined by Zhang *et al.* [23].

Statistical Methods

The histological result was applied as the reference standard in assessing the predictive value of the physical status in cervical cancer development using the cytological result as baseline. Mann-Whitney and Kruskal-Wallis non-parametric methods were used to discriminate differences in HPV DNA physical status among the different cytological and histological categories. Student's *t*-test

analysis was performed to measure the difference between the mean E2/E6 ratios for both genotypes through the clinical diagnosis categories. A two-sided P value ≤ 0.05 was considered statistically significant. All analyses were performed by using the IBM SPSS Statistics version 20.0 (IBM Corporation Inc., Armonk, NY, USA).

RESULTS

Regarding the real-time PCR methodology used for calculating E2/E6 ratio, both sets of primers have demonstrated specificity for HPV 16 and HPV 18 E2 and E6 genes, as no cross-reaction was observed for samples harboring other high-risk HPV genotypes. The standard curve evidenced a dynamic linear range with very good correlation ($R^2 > 0.9914$ for HPV 16 and $R^2 > 0.9935$ for HPV 18). For both genotypes, the PCR amplification efficiencies for E2 and E6 genes were > 92.3%.

Concomitant forms of HPV 16 were observed in 74.0% (267/361) of the cases whereas it was episomal or linear in 19.7% (71/361) and 6.4% (23/361) of the cases, respectively. The mean age of patients infected with HPV 16 episomal forms (32.4 ± 11.3 years) was lower than the determined for the HPV 16 linear forms (36.5 ± 11.5 years), but no statistical significance (P = 0.073) could be established (Fig.1).

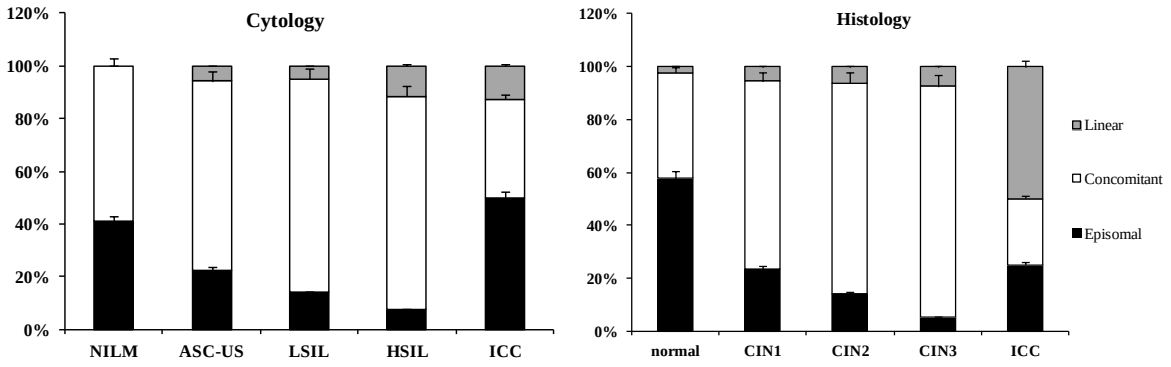

Fig. 1: Distribution of HPV 16 DNA physical status by cytological and histological grade. The percentage bars are presented with standard error bars. NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma; CIN, cervical intraepithelial neoplasia grade 1 to 3.

Considering the clinical diagnosis, the presence of HPV 16 linear forms increased with increasing disease grade, while episomal forms were more frequently detected in normal cytologies (NILM) (41.0%; 32/78), and the concomitant forms, although detected in a higher proportion (>70.0%), their detection was independent of disease grade (P = 0.232) (Fig.1). As for histological results, 57.8% (26/45) of the HPV 16 episomal forms were detected in negative histologies, and their frequency diminished with increasing disease grade (only one out of four ICC cases). Concomitant

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forms were highly detected independently of the disease grade but they were more frequent among CIN lesions (> 70%), and they were only present in one out of four ICC cases (P = 0.006). Concerning E2/E6 ratio, no differences were found between normal histologies and CIN3 lesions (0.632 ± 0.236), whereas a significant association was identified between low E2/E6 ratios and ICC (P < 0.001) (Fig.2).

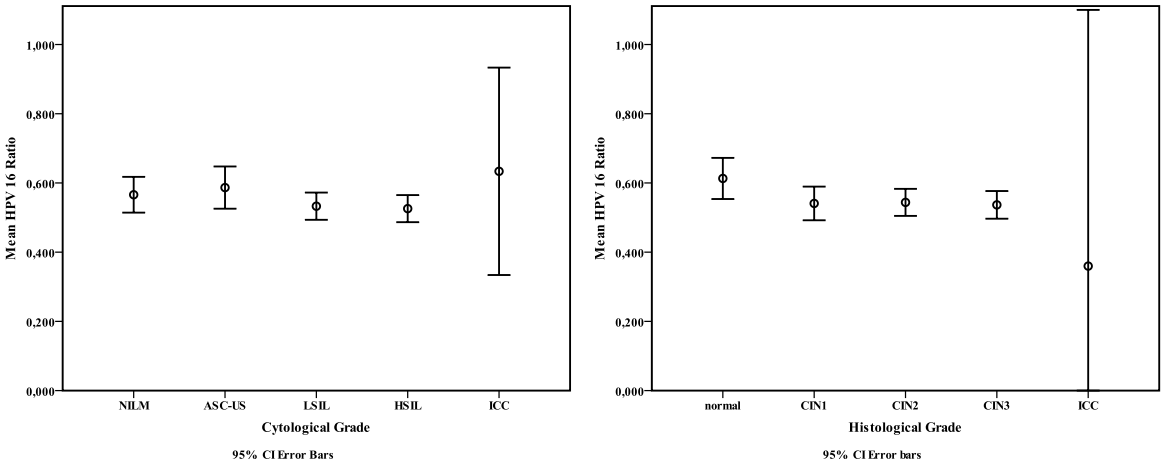

Fig. 2: Comparison of HPV 16 E2/E6 ratio according to the different clinical diagnosis. Data is presented as T-plots, representing the 5th and 95th percentiles. NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC Cervical carcinoma; CIN, cervical intraepithelial neoplasia grade 1 to 3.

Concomitant forms of HPV 18 were observed in 55.8% (77/138) of the cases whereas it was linear or episomal in 31.2% (43/138) and 13.0% (18/138) of the cases, respectively. The mean age of patients infected with HPV 18 episomal forms (32.6 ± 9.7 years) was lower than the determined for HPV 18 linear forms (33.8 ± 10.3 years), but no statistical significance (P = 0.393) could be established (Fig.3).

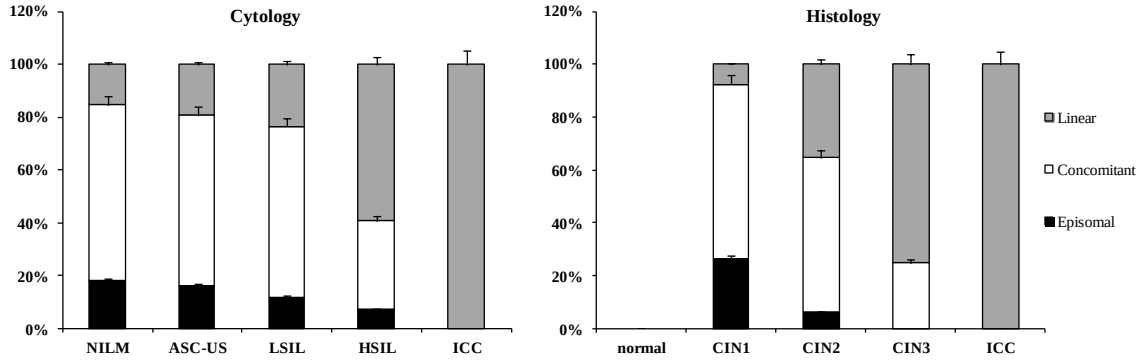

Fig. 3: Distribution of HPV 18 DNA physical status by cytological and histological grade. The percentage bars are presented with standard error bars. NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma, including adenocarcinoma; CIN, cervical intraepithelial neoplasia grade 1 to 3.

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Considering the cytological diagnosis, the concomitant form was the most frequently detected in every lesion group (> 60.0%) whereas the episomal was detected in about 15.0% of the cases from each cytological category. There was an increasing proportion of linear forms detected according to the severity of lesion grade, reaching 100.0% among adenocarcinoma cases ($P < 0.001$; Fig.3). As for histology results, 26.4% (14/53) of the episomal forms were detected in CIN1 cases, decreasing to 6.2% (4/65) among CIN2 cases and none was detected in CIN3 lesions or ICC cases. The proportion of concomitant forms also decreased from 66.0% (35/53) to 25.0% (4/16) among CIN1 to CIN3 lesions, respectively. In opposition, the proportion of linear forms increased from 7.5% (4/53) in CIN1 lesions and 35.4% (23/65) in CIN2 lesions, to 75.0% (12/16) among CIN3 lesions; all adenocarcinomas (n = 4) presented linear HPV 18 forms ($P < 0.001$; Fig.3). Considering E2/E6 ratio, there was a significant linear decrease of the ratio associated with the severity of lesion grade (0.054 ± 0.112; $P = 0.001$) (Fig.4).

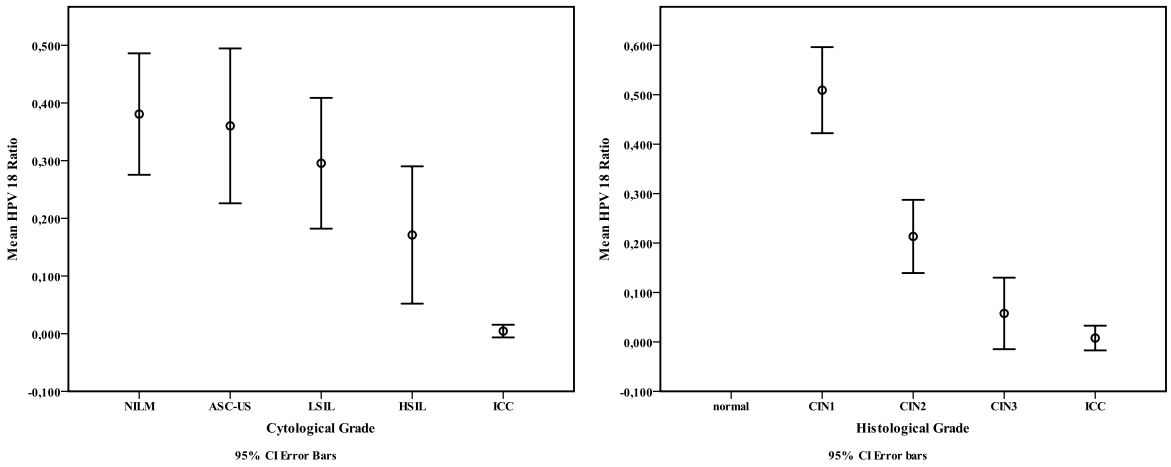

Fig. 4: Comparison of HPV 18 E2/E6 ratio according to the different clinical diagnosis. Data is presented as T-plots, representing the 5th and 95th percentiles. NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC Cervical carcinoma, including adenocarcinoma; CIN, cervical intraepithelial neoplasia grade 1 to 3.

DISCUSSION

HPV DNA physical status has been claimed to be a useful biomarker, namely due to its prognostic significance for cervical cancer development, and it might be predictive of lesion progression among women infected with high-risk HPV genotypes [13,14]. This study aimed to evaluate the physical status of HPV 16 and 18 DNA as a biomarker for cervical cancer development. To fulfill this purpose, a previously described real-time quantitative PCR approach was used [21,22]. Although the real-time PCR methodology applied in the present study may be considered a suitable methodology for the management of HPV-infected women, special attention should be addressed to circulating variants of oncogenic HPV genotypes. In fact, during preliminary stages of the present study, the HPV 16 physical status determination required some adjustments regarding the set of

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primers, as real-time PCR failed to detect the cervical cancer-associated African and Asian-American variants [24], further identified by E6 gene sequencing. Some authors reported a PCR in-house approach [25-28], followed by agarose gel analysis, which, in comparison with the adopted methodology, has demonstrated to be subjective and far less specific. Also, PCR in-house procedures are laborious, time-consuming and require large quantities of DNA to correctly estimate the physical status of the viral DNA, in contrast to real-time quantitative PCR.

Real-time PCR methodologies have demonstrated to be adequate for the determination of HPV DNA physical status, namely when using paraffin-embedded tissues for which the major problem is the use of a correct DNA extraction procedure. However, these methodologies fail to distinguish the number of episomal genomes from integrated viral genomes. HPV DNA integration is thought to be a single event that occurs surrounded by a probable high number of episssomal genomes, which may influence the sensitivity of real-time PCR methodologies in detecting integrated forms [29,30]. Even so, the E2/E6 ratio may provide a fairly good estimation of this relation if the exclusively episomal forms (quantitation of E2 gene) are subtracted from the overall quantitation of E6 gene (corresponding to integrated forms + episomal forms). In the present study, real-time PCR confirmed to be an appropriate methodology since no cross-reactions were observed in multiple infections involving HPV 16 and/or 18, and other high-risk HPV genotypes. Furthermore, by applying the histological diagnosis as the gold-standard for assessment of the prognostic significance of the 'physical status' biomarker, it is possible to understand the involvement of this event in cervical disease through a correspondence between the integrated genomes and the histologically confirmed high-grade lesions. The cytological diagnosis in which the HPV DNA physical status is determined provides useful information regarding its potential use for diagnostic purposes in a clinical setting.

High-risk HPV is often found integrated into the host cell genome, which is considered critical for the malignant transformation of host cells. Some authors [17,20,21] have shown that HPV integration occurs at the early stages of cervical cancer development, as this biological feature can be detected in all CIN stages. Studies focused on HPV 16 showed that the proportions of linear DNA increased with lesion grade; however, for other carcinogenic HPV data is still scarce, namely for HPV 18 [15,31-34].

In the present study, the percentage of concomitant and linear forms (82.2%; 410/499) for both genotypes is consistent with the described by other authors [14,15,17,21,32,34], and it corroborates, especially for HPV 16, that integration may be an early event during carcinogenesis. In fact, concomitant forms were the most frequently detected, independently of the disease grade. Yet, the fact that even in CIN2 cases or worse episomal forms were detected could indicate that HPV 16 integration might not be essential for malignant transformation. Nonetheless, only eight cases of ICC were studied, among which one presented the HPV 16 DNA in the linear form, whereas the remaining seven presented the DNA whether in concomitant or episomal forms. Considering the

mean E2/E6 ratio, the values were similar independently of the severity of the cervical lesion, from normal histology to CIN3 lesions; however, among ICC cases the E2/E6 ratio was significantly lower.

Previous studies [26,32] determined that the frequency rate of exclusively episomal forms of HPV 18 ranged from 9.8% and 36.7% in ICC. These findings suggest that unknown factors, other than integration, are likely to be involved in the induction or maintenance of HPV 18 E6 expression during cervical carcinogenesis. However, and despite the low number of adenocarcinoma cases involved in the present study, none presented the episomal form, which agrees with the higher aggressiveness of HPV 18 previously proposed by Woodman *et al.* [35]. On the other hand, the proportion of HPV 18 linear forms increased significantly with the disease grade and a linear relation between E2/E6 ratio and disease grade was observed, as evidenced by a shift from the exclusively episomal form to the exclusively linear form according to the severity of the disease. In fact, integration of the HPV 18 DNA into the host cell appears to be a crucial and determining event during cervical carcinogenesis, strongly associated to cervical cancer development. All together, more significantly than to HPV 16, the determination of the physical status of HPV 18 DNA constitutes a useful biomarker, as it could predict the risk of cervical cancer development, where lower E2/E6 ratios would be clinically associated with prevalent precancerous lesion.

Considering the systematic analysis proposed by Saunier *et al.* [17] in which a cut-off value for E2/E6 ratio lower than 0.520 represents a higher risk for the development of high-grade lesions, in this study, 35.2% (127/361) of the HPV 16-positive and 63.8% (88/138) of HPV 18-positive cases identified through the present study would be at risk of cervical cancer development. The majority of these cases at risk corresponded to high-grade cervical lesions (CIN2 or worse) for both genotypes, meaning that there is a good correlation between the determination of the HPV DNA physical status and the histological diagnosis. In a clinical scenario where low-grade cervical lesions might regress spontaneously within the first years of infection, it is important to rationally manage HPV 16/18-positive women, avoiding unnecessary aggressive treatments, which could increase morbidity, but rather improve diagnosis and risk assessment, namely by using molecular markers as the evaluated in this study. However, research on the mechanisms and corresponding biomarkers associated with neoplastic progression, remains an important priority for future screening test improvements.

The findings reported here clearly evidence a tendency where HPV 18 DNA integration may be a crucial event in malignant cervical cancer development, and for this reason, women presenting integrated DNA together with abnormal cytology should be closely monitored. Regarding HPV 16 integration, it seems to be an early event and its involvement in the malignant transformation of host cells is less suggestive, which could mean that other virological, host or environmental factors should play a more effective role on cervical cancer development.

Despite some limitations of the present cross-sectional study, that precludes the determination of the longitudinal clinical relevance of HPV physical status as a biomarker, namely

the low number of ICC cases, attention should be addressed to HPV 18 physical status as a predictive biomarker of cervical cancer for future patient management and cervical cancer prevention, helping to distinguish the clinical relevance of HPV infection. These limitations evidence the need for larger studies involving women infected by the different HPV carcinogenic genotypes at different clinical stages.

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Prognostic value of human papillomavirus types 16 and 18 DNA physical status in cervical intraepithelial neoplasia

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Abstract

The aim of this work was to assess the value of the physical status of human papillomavirus (HPV) DNA as a disease marker for cervical cancer development in a set of 248 DNA samples previously genotyped as HPV 16 or 18, by calculating the E2/E6 ratio through real-time PCR. There was a significant difference in integration status according to disease grade for both genotypes ($p < 0.001$). Furthermore, especially for HPV 18, determining the DNA physical status could be a useful biomarker in predicting cervical cancer risk development, with a lower E2/E6 ratio clinically associated with the development of a precancerous lesion.

Keywords: Cervical cancer, human papillomavirus 18, molecular marker, physical status, risk assessment
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Almost all cervical cancers are related to human papillomavirus (HPV) infection, and HPV 16 and 18 genotypes are responsible for more than 70% of the cases worldwide [1 3]. HPV 18 is more frequently detected in adenocarcinoma and adenosquamous carcinoma, whereas HPV 16 is more associated to squamous cell carcinoma [4,5]. In Portugal, HPV 16 is the most frequently detected genotype by cytological grade (19.7%); HPV 18 has a low prevalence (4.4%) [6].

Integration of HPV into the host genome is a prerequisite for the development of malignant lesions, resulting from a disruption in the HPV genome through partial loss of the E2 gene [7,8]. In addition, recent data suggest that integration frequency in premalignant and malignant lesions varies with HPV genotype [5,9]. Physical status of HPV DNA (episomal, linear or concomitant/mixed forms) has also been considered as a marker of disease progression [10 12]. Absolute quantification of E2 and E6 genes by real-time PCR, followed by calculation of the E2/E6 ratio has been proposed [13], allowing identification of the different forms of the viral DNA. The aim of this study was to investigate these associations regarding HPV 18 in comparison with HPV 16 cases.

The study group comprised 248 frozen DNA aliquots of cervical samples, from women aged 18 65 years (mean age 33.1 ± 9.3 years; median 31 years), genotyped previously as HPV 16 ($n = 132$) or HPV 18 ($n = 116$) by CLART HPV2 (Genomica, Madrid, Spain). The cervical samples were collected in ThinPrep PreservCyt medium (CyticUK, Crawley, UK) for cytological analyses. Histological evaluation made on colposcopically directed biopsies was also available (Table 1). The histological result was applied as the reference standard in assessing the predictive value of the physical status in cervical cancer development using the cytological result as baseline.

The physical status of HPV 16 and 18 DNA was assessed by real-time PCR amplification of E2 and E6 genes, as described previously [14,15]. Real-time PCR was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For each sample, absolute quantification of E2 and E6 genes was performed in duplicate, simultaneously in the same plate.

Mann Whitney and Kruskal Wallis non-parametric methods were used to discriminate differences in DNA physical status among the different cytological or histological categories. Student's *t*-test analysis was performed to measure the difference between the mean E2/E6 ratios for both genotypes through the clinical diagnosis categories. A p value ≤ 0.05 (two-sided) was considered statistically significant. All analyses were performed using the IBM SPSS Statistics version 20.0 (IBM Corporation Inc., Armonk, NY, USA).

Among the HPV 16-positive samples, 40.2% (53/132) were episomal, 58.3% (77/132) presented concomitant forms, and 1.5% (2/132) were in the linear form. The episomal forms were more frequent in normal cytologies, whereas the concomitant forms increased according to disease grade (p 0.011; Fig. 1a). An increasing proportion of concomitant forms was identified

TABLE 1. Characteristics of the studied samples

	Cases (%) (n = 248)	HPV 16-positive (%) (n = 132)	HPV 18-positive (%) (n = 116)
Cytology			
NILM	79 (31.8)	42 (31.8)	37 (31.9)
ASC-US	56 (22.5)	26 (19.7)	30 (25.9)
LSIL	66 (26.6)	35 (26.5)	31 (26.7)
HSIL	41 (16.5)	27 (20.5)	14 (12.1)
ICC	6 (2.4)	2 (1.5)	4 (3.4)
Histology			
Normal	30 (12.1)	30 (22.7)	0 (0.0)
CIN1	97 (39.1)	47 (35.6)	51 (44.0)
CIN2	99 (39.9)	44 (33.3)	54 (46.6)
CIN3	16 (6.5)	9 (6.8)	7 (6.0)
ICC	6 (2.4)	2 (1.5)	4 (3.4)

NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma, including adenocarcinoma; CIN, cervical intraepithelial neoplasia grade 1–3.

from normal histology through to cervical intraepithelial neoplasia (CIN) grade 3 lesions, ranging from 33.3% (10/30) to 88.9% (8/9), respectively ($p < 0.001$; Fig. 1b). Concerning the E2/E6 ratio, no significant difference was found between normal histologies and CIN2 cases (0.959 ± 0.112) ($p < 0.001$; Fig. 2a).

Among the HPV 18-positive cases, 13.8% (16/116) were episomal, 65.5% (76/116) presented concomitant forms, and 20.7% (24/116) were in the linear state. Cytologically, the episomal forms were detected in 15% of the cases, whereas the concomitant form was the most frequent in every lesion group (>60.0%). An increasing proportion of linear forms was related to the severity of the lesion grade (p 0.029; Fig. 1c). The proportion of concomitant forms decreased from 72.5% (37/51) to 28.6% (2/7) in CIN1 and CIN3 lesions, respectively, whereas the proportion of linear forms increased from 24.1% (13/54) in CIN2 lesions, to 71.4% (5/7) in CIN3 lesions, and 100% (4/4) in adenocarcinomas (ICC) ($p < 0.001$; Fig. 1d). Considering the E2/E6 ratio, there was a significant linear decrease in the value of the ratio associated with the severity of lesion grade (0.054 ± 0.112; $p < 0.001$) (Fig. 2b).

The percentage of concomitant and linear forms (72.2%; 179/248) is consistent with other studies [8,13,14]. As expected, especially for HPV 16, our data showed that integration may be an early event during carcinogenesis, as concomitant forms were the most frequently detected [8,12,13,16 18]. Yet, the fact that even in CIN2 cases episomal forms were detected could indicate that apart from HPV 16 integration, other mechanisms might lead to carcinogenesis. Considering the mean E2/E6 ratio in the histological diagnosis, values were similar from normal histology to CIN2 lesions, and only in CIN3 lesions the mean E2/E6 ratio was considerably lower.

Previous studies determined that the frequency rate of exclusively episomal forms of HPV 18 ranged from 9.8% to 36.7% in ICC [18,19]. However, in our study no episomal form

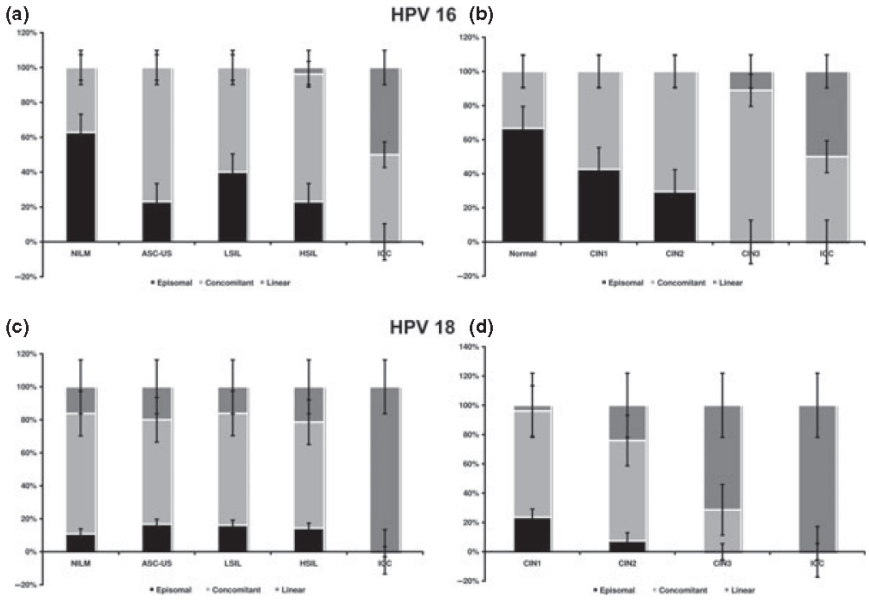

FIG. 1. Physical status of viral DNA from (a) human papillomavirus (HPV) type 16 according to cytology; (b) HPV 16 according to histology; (c) HPV 18 according to cytology; and (d) HPV 18 according to histology. Data are presented with standard error bars. NILM, normal cytology; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; ICC, cervical carcinoma, including adenocarcinoma; CIN, cervical intraepithelial neoplasia grade 1–3.

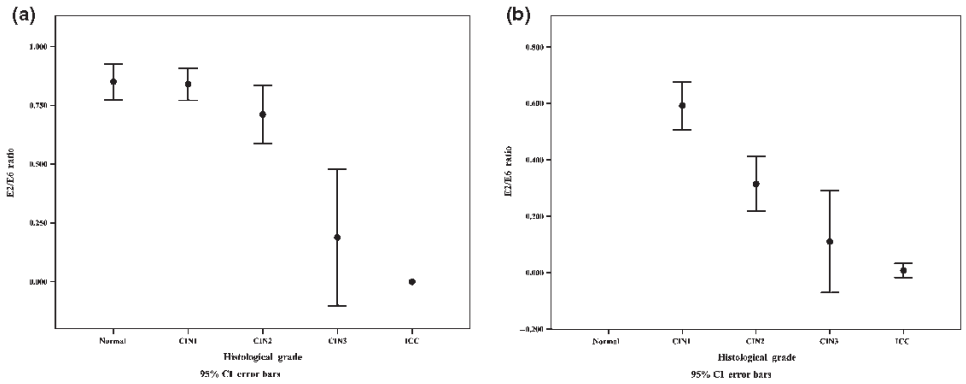

FIG. 2. Comparison of human papillomavirus (HPV) type 16 (a) and HPV 18 (b) E2/E6 ratio according to the different histological categories. Data are presented as T-plots, representing the 5th and 95th centiles. Normal, normal histology; CIN, cervical intraepithelial neoplasia grade 1–3; ICC, cervical carcinoma, including adenocarcinoma.

was present in adenocarcinomas, suggesting a more aggressive biological potential of this genotype, as previously stated [5]. In fact, we observed a relation between HPV 18 E2/E6 ratio and disease grade, as shown by a shift from the exclusively episomal form to the exclusively linear form according to the severity of the lesion.

Regarding the natural history of HPV, it is important to manage HPV 16-positive or HPV 18-positive women by improving diagnosis and taking into account type-specific characteristics associated with malignant transformation [5,12]. Considering the systematic analysis proposed by Saunier *et al.* [8], in which a cut-off value for E2/E6 ratio lower than 0.520 represents a higher risk for the development of high-grade lesions, in this study, 16.7% (22/132) of HPV 16-positive and 55.2% (64/116) of HPV 18-positive women were at risk.

This study has some limitations. First, it is a cross-sectional study, precluding the determination of the longitudinal clinical relevance of this biomarker. Also, the low number of high-grade lesions included in this study shows the need for further studies to achieve significance in women with cervical cancer. Further evaluation of the determination of HPV 18 physical status as a predictive biomarker must be addressed. Nonetheless, HPV physical status, especially in women infected with HPV 18, seems to be important in patient management and cervical cancer prevention, helping to distinguish the clinical relevance of HPV infection.

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Transparency Declaration

The authors declare that there are no conflicts of interest.

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CHAPTER 9

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Contributions of the Authors:

Ana Gradíssimo was responsible for the research, writing and organization of this paper. She was also responsible for all the mathematical modeling and all the data analysis, including figures and tables.

Miguel Abreu was responsible for assembling the mathematical formula.

Nuno Verdasca contributed with proofreading and image rearrangements.

ORIGINAL ARTICLE

Mathematical Modeling in HPV Infection: A Simplified Approach

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Running Title: Mathematical Model Applied to HPV Infection.

ABSTRACT

Cervical cancer prevention strategies are based on assumptions and projections of estimates for HPV infection dynamics. In this context, mathematical models provide a useful tool to estimate scenarios and strategies effectiveness. Here, the aim was to develop a mathematical model applicable to genital HPV infection, to further estimate different HPV infection scenarios based on opportunistic and routine epidemiological data, and to formulate predictions putatively useful for the implementation of cervical cancer prevention strategies more adequate to Portuguese women. A simple compartmental Markov model was developed comprehending six mutually exclusive disease states in order to simulate a simplified path of the HPV natural history towards cervical carcinogenesis. The evolution was explored in an opportunistic screening-type population (Chapter 3) and compared with a routine screening-type population (CLEOPATRE Study). This study design led to an initial stratification of all women through the various disease states at the entry of the model. Different scenarios of HPV vaccination coverage rate (85% and 100%) for women aged 18 to 25 years were also simulated. The predicted progression curves evidenced a 32.5% decrease on HPV 16 and 18 infections for the 'Study Population', and a decrease also on HR HPV infections, with a corresponding lower proportion of cervical lesions, contributing to a decrease of around 10% in high-grade cervical lesions. The estimates also evidenced some impact of routine screening programs over cervical cancer prevention rates (60.0%).

Keywords: Cervical cancer, human papillomavirus, prevention strategies, mathematical modeling.

Not Published.

INTRODUCTION

Human papillomavirus (HPV) infection is a determinant factor for the development of cervical cancer [1,2]. As reported from the International Agency for Research on Cancer (IARC), the oncogenic HPV 16 and 18 are the most prevalent genotypes associated to invasive cervical cancer (ICC) cases [3-5]. In addition, HPV contributes to the development of numerous cancer-related diseases, including anogenital carcinomas (vulvar, vaginal, anal, penile) [6-8], as well as head and neck squamous cell carcinomas [9-12], where HPV 16 and 18 are also the most carcinogenic genotypes. Also, highly recurrent benign diseases, such as genital warts, are caused by HPV 6 and 11 genotypes (almost 90.0% of the cases) [12,13].

Two vaccines against HPV infections have been released, namely Gardasil® (Merck & Co., Inc., Whitehouse Station, NJ, USA) in 2006, often referred to as the quadrivalent vaccine and Cervarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium) in 2007, also referred to as the bivalent vaccine. Gardasil® targets the oncogenic HPV 16 and 18 genotypes and two non-oncogenic HPV genotypes (6 and 11). Cervarix® targets the same two oncogenic HPV genotypes (16 and 18) but no non-oncogenic HPV genotype, and is formulated with a novel adjuvant, ASO4, which is intended to boost the immune response [14,15]. For Cervarix®, the findings from randomized clinical trial studies evidenced an efficacy of 100% in preventing HPV 16/18-associated cervical intraepithelial neoplasia (CIN) lesions [16-18]. For Gardasil®, the efficacy is greater than 90.0% in preventing HPV 6/11/16/18-associated CIN lesions, adenocarcinoma, genital warts, or vulvar and vaginal intraepithelial neoplasia (VIN or VaIN, respectively) [19].

Cervical cancer prevention strategies are dependent of country-specific information regarding the dynamics of HPV infection, so that epidemiological data is essential for the formulation of adequate measures and prevention programs, including HPV vaccination programs, regardless of vaccines clinical efficacy and safety [20,21]. In this context, mathematical models constitute useful tools to estimate scenarios and strategies effectiveness. Through the combination of results from randomized clinical trials, and from biologic and epidemiologic studies, mathematical models would be able to predict and address the main questions underlying each HPV infection prevention strategy. However, these models are based on assumptions and estimates, which may bring uncertainty [19]. Regarding HPV infection, this uncertainty is of some importance, mainly due to the complexity of the model, relying on the natural history of HPV infection, which includes several stages of infection and disease progression that are variable within each specific genotype. Also, the baseline information that would define model parameters is difficult to estimate; in fact, studies on age- and genotype-specific epidemiology and transmission rates are still scarce. Moreover, as it was considered for HPV vaccine randomized clinical trials, the use of ICC cases to estimate progression rates cannot be carried out for ethical reasons.

Epidemiological data on HPV infection among Portuguese women have been recently published [22,23]. One study involved normal cytology cases from routine screening-like population [22], while the other was a retrospective study focused on HPV genotype-specific detection in precancerous lesions and cervical cancer cases [23]. In the present study, the aim was to develop a mathematical model applied to genital HPV infection, to further estimate different HPV infection scenarios based on epidemiological data from opportunistic and routine settings, and to formulate predictions putatively useful for the implementation of cervical cancer prevention strategies more adequate to Portuguese women.

MATERIAL AND METHODS

Study Design

The model was developed to be applied on two different populations, one involving characteristics of opportunistic screening ('Study Population') and another involving routine screening ('Reference Population') of women sexually active aged 18 to 65 years. The 'Study Population' was composed by a cohort of 2149 women distributed along the defined diseases states of the model at baseline according to data collected in Chapter 3. The 'Reference Population' corresponded to a cohort of 2326 women that participated in the CLEOPATRE Portugal Study [22], and were also distributed along the disease states of the defined model. For the purposes of the aimed model, it was considered that both populations would be followed through their lifetimes.

Additionally, the simulations were carried out on a theoretical population ('Hypothetical Population') which consisted in 2149 women (for approximation to 'Study Population' scenarios), aged 18 years and HPV-negative at baseline, to explore the maximum impact of HPV vaccination in a population not exposed to HPV infection (approximately 1.0% of young female population vaccinated).

Model structure

A simple compartmental model of the natural history of HPV infection into ICC was developed incorporating transitions between five mutually exclusive disease states, defined to describe each woman's clinical condition: HPV-negative (susceptible; 'HPV-ve'); HPV-positive (infected); low-grade cervical lesion ('LSIL'); high-grade cervical lesion ('HSIL'); and 'ICC'. The 'HPV-positive' state was further divided into two different states: HPV 16/18-positive ('HPV 16/18+ve'); and high-risk (HR) HPV-positive ('HR HPV+ve'), to incorporate estimates of HPV genotype-specific infection rates. Low-risk HPV infection was ignored in the model because the previous determined proportion was too low (4.0 %) (Chapter 3) and it was associated with HR HPV infection in the vast majority of the cases. In addition, for each incremental year in the model, the

cohort was subjected to an age-dependent probability of acquiring HPV. Viral infection was defined proportionally as either detectable ('HPV-positive') or undetectable ('HPV-negative'), and disease states were defined using the Bethesda classification system (SIL lesions) [24,25]. Similarly to what is applied to Markov models, in this study, the conditional distribution of the outcomes given a disease state depends on prior outcome observations only.

To define model parameters, several data were collected across different studies. The transition probabilities used to "move" women through the disease states over time were extrapolated from published studies [20,24,26-28], and further explained in the following sections. Whenever deemed necessary, the probabilities were adjusted by the modeler on the assumption that each probability is disease state-specific; however, this adjustment may carry some bias to the model. The time lapse defined to run the model was 30 years (arbitrary decision of the modeler) and was divided into equal increments per year, referred to as Markov cycles, during which women transitioned from one disease state to another. Each year, women may get infected with HPV (any of the sub-groups previously defined), develop SIL lesions, progress to a higher grade SIL or cancer, regress from a higher to a lower grade SIL or normal epithelium (infected state), or clear the infected state. In each cycle, women can die from any cause other than cervical cancer ('Dead') (age- and sex-related all-cause mortality adjusted rate) [29].

Simulations were carried out using R software version 2.12.1 (R Project for Statistical Computing, http://www.r-project.org/). The predictive outcome of the model was assessed by the generation of age-specific progression curves predicted over time for HPV 16/18 infection, HR HPV infection, LSIL and HSIL lesions, for further comparison with published data.

The model was used to address the following questions:

1. Given the current status of the 'Study Population', the most effective approach would be to prevent HPV infection or to treat clinical symptoms?
    a. Which would be the evolution for the 'Study Population', without any clinical procedure?
    b. To what extent HPV vaccination would contribute to the prevention of HPV-associated cervical lesions in the 'Study Population'?
    c. How does HPV vaccination impact on the prevention of HPV 16 and 18 infections in the 'Study Population' for a coverage rate of 85%?
2. For each disease state, what would be the main differences between routine screening-based and opportunistic screening-based populations?
    a. How would the routine screening impact on HPV infection dynamics for a 'Reference Population'?
    b. Different coverage rates (85% *versus* 100%) of HPV vaccination would impact on the 'Reference Population'?
3. How would be HPV vaccination effective in a population not exposed to HPV infection?

To address question 1, different scenarios were estimated for the population studied in Chapter 3 comprehending no clinical intervention (question 1a), or the possibility to vaccinate women below 25 years without HPV 16 and 18 infection (questions 1b and 1c) for a coverage rate of 85% [30] or 100%. These scenarios were further evaluated to propose the best strategy to provide effective health-care to the 'Study Population'. The outcomes were based on the estimates of the proportion of HPV infection (in the two sub-groups) and the proportion of HPV-associated LSIL, HSIL and ICC cases for the overall population. At baseline, women entered the model at their current disease state, as determined in Chapter 3.

To address question 2, the simulations were carried out in a 'Reference Population' from an epidemiological study in Portugal (CLEOPATRE Study), including non-vaccination (question 2a), or the possibility to vaccinate women below 25 years without HPV 16 and 18 infection (question 2b) for a coverage rate of 85% and 100%. The estimates were further compared with the estimates obtained for the 'Study Population' to assess the main differences between the two populations.

To address question 3, a theoretical population, as referred in the Study Design, was tested to assess differences for the HPV vaccination impact regarding the 'Study Population'.

Natural History

Contrary to other studies [20,26-28,31,32], women from both populations were distributed along the different disease states at baseline, assuming different proportions in the beginning of the simulations. Several conditions were assumed as static, while others were allowed to vary, based on epidemiological cohort published data:

- All cases of cervical cancer depend on a prior HPV infection and on a prior cervical lesion (LSIL and HSIL), and sequential transitions are obligatory between disease states through the designed model, given that HPV is the causative agent for cervical cancer [1,2].
- Clearance of HPV infection is defined as an undetectable HPV DNA result following a previous detection of an HPV genotype in the same woman by using the same diagnostic test.
- Acquisition of HPV infection was based on age-specific incidence rates and defined into two age groups (18-25 and 26-65 years), assuming that young women tend to present higher infection rates [33,34].
- Clearance time of HPV infection was defined as age-specific into two age groups (18-25 and 26-65 years), where young women present no detectable HPV DNA in almost 90.0% of the cases after two years upon initial infection [35].
- Clearance rates are dependent of specific genotypes, v.g. HPV 16 presents a longer persistency of infection and consequently a higher risk of SIL development (around 40.0% in five years) [36]. This supports the evidence that HPV genotype is the most significant risk

factor for cervical cancer development, as the majority of HSIL lesions and ICC cases are associated to high-risk HPV infection, namely HPV 16 and 18 [5].

- Acquisition of HPV infection and SIL development was defined as genotype-specific and was divided into two groups ('HPV 16/18', and 'HR HPV'), given the increased risk linked to HPV 16 and 18. Additionally, this stratification allowed the assessment of the impact of HPV vaccination for the populations under evaluation.
- Transition probabilities for developing LSIL and HSIL lesions were defined as age-specific into two age groups (18-25 and 26-65 years), since young women present higher incidence estimates for SIL lesions (HSIL is identified after two years of the onset of an high-risk HPV infection) [35,37,38]. In addition, young women present lower rates of progression from SIL lesions to ICC cases (approximately one percent per year) [39,40].
- ICC development rates were assumed as 12.0 to 31.0% of precancerous lesions (if left untreated) [39,41,42].

Vaccination

The base-case scenario of the assumed genital HPV infection model includes a simulation of non-vaccination, which was further compared to additional scenarios of vaccine coverage rates (85% and 100%), to explore the impact of HPV vaccination in both women populations. The possibility to be vaccinated was only allowed by the modeler to women aged below 25 years. Estimates were generated considering the overall population and further comparisons were performed comprehending three different HPV vaccination scenarios (0%, 85% and 100%) within each population. Predictions were estimated based on the following assumptions: i) vaccine duration is lifelong; ii) 100% reduction in susceptibility to HPV 16 and 18 infections (vaccine efficacy); and iii) variable proportion of women protected following vaccination, coverage level, of 100% or 85.0% (as observed by the General Directorate of Health (DGS) in Portugal – Chapter 2) [30]. Moreover, no cross-protection data was included, regarding protection conferred from HPV vaccines to prevent infection by other HR HPV genotypes.

RESULTS

Overview

All the possible transitions that correspond to HPV natural history are depicted in Fig.1, with the assumed transitions represented by solid lines and the ignored transitions by dotted lines, constituting the final genital HPV infection model assumed for the simulations.

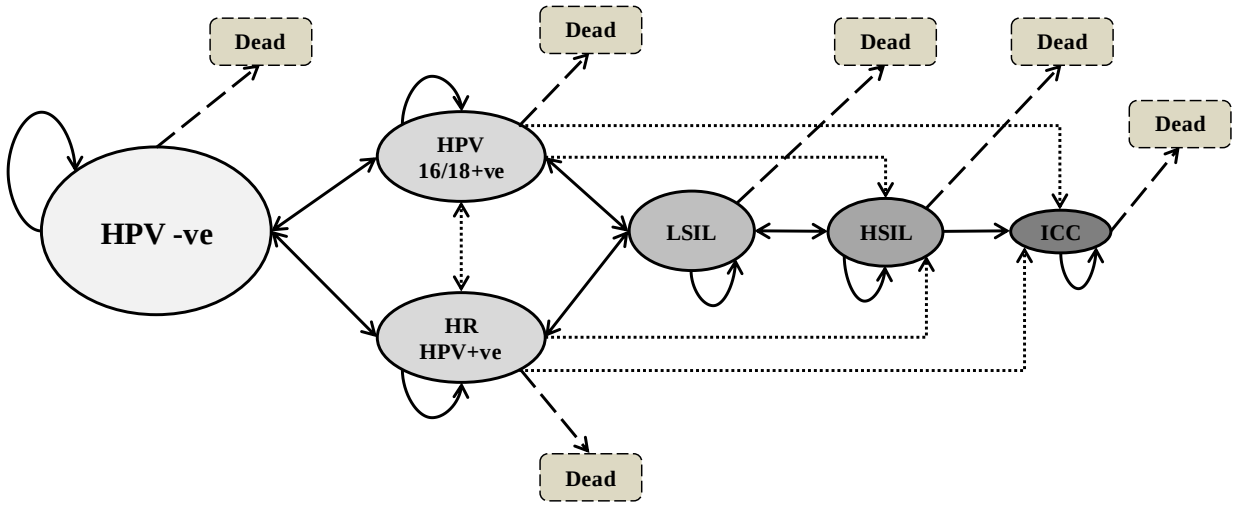

Fig.1: Genital HPV infection model diagram. The model assumes the inclusion of six mutually exclusive disease states: 'HPV-ve', HPV-negative; 'HPV 16/18+ve', HPV-positive for genotypes 16 and 18; 'HR HPV+ve', HPV-positive for high-risk genotypes; 'LSIL', low-grade cervical lesions; 'HSIL', high-grade cervical lesions; 'ICC', cervical cancer. For all disease states is included an age-dependent probability of death by causes other than cervical cancer ('Dead' state) (dashed lines). Disease states 'ICC' and 'Dead' are called as absorbing states, as women remain in this states for the remainder of the simulations. The transitions represented in solid black lines correspond to the transitions included in the simulations, whereas the dotted black lines correspond to transitions not included in the simulations. The arrows represented in both ways correspond to acquisition *vs* clearance of HPV infection (all sub-groups), or progression *vs* regression of cervical lesions. The circular arrows correspond to the probability to remain in the same disease state in one Markov cycle (one year). No transitions were allowed between the two different genotype-specific sub-groups.

Formula and Parameters

The model was based on the following formula:

$$X_j^{[a+1]}(T+1) = \sum_{i=1}^{m} p_{ij}^{[a]}\ X_i^{[a]}(T)$$

Where $X_i^{[a]}(T)$ is the number of women in age group $[a]$ included in the disease state i for the time T (measured in years); whereas: $i = 1, \ldots, m = 7$ (as the number of disease progression states defined).

The transition probabilities are defined as $p_{ij}^{[a]}$ that corresponded to the annual probability for one woman to transition from disease state i to disease state j (and $\sum_{j=1}^{m} p_{ij}^{[a]} = 1$, for each age group $[a]$).

The calculations were carried out as follows:

$$\left[X_1^{[a+1]} \quad \ldots \quad X_m^{[a+1]}\right] = \left[X_1^{[a]}(T) \quad \ldots \quad X_m^{[a]}(T)\right] \times \begin{bmatrix} p_{11}^{[a]} & \cdots & p_{1m}^{[a]} \\ \vdots & \ddots & \vdots \\ p_{m1}^{[a]} & \cdots & p_{mm}^{[a]} \end{bmatrix}$$

The probabilities used in the transition matrixes are presented in Table 1.

Table 1: Transition probabilities included in the genital HPV infection model

Transition	Probability			Reference
	Acquisition	Clearance	Maintenance	
'HPV-ve' vs 'HPV 16/18+ve'	0.0980 (18-25 years) 0.0500 (26-65 years)	0.5297 (18-25 years) 0.2797 (26-65 years)	-	
'HPV-ve' vs 'HR HPV+ve'	0.1140 (18-25 years) 0.0570 (26-65 years)	0.6297 (18-65 years)	-	[26,34,36,37,43-45]
'HPV-ve'†	-	-	Assumed	
'HPV 16/18+ve'†	-	-	0.2500 (18-65 years)	
'HR HPV+ve'†	-	-	0.2000 (18-65 years)	
	Progression	**Regression**	**Maintenance**	
'HPV 16/18+ve' vs 'LSIL'	0.0900 (18-25 years) 0.3500 (26-65 years)	0.9000 (18-65 years)	-	
'HR HPV+ve' vs 'LSIL'	0.0900 (18-25 years) 0.0600 (26-65 years)		-	[46-48]
'LSIL' vs 'HSIL'	0.0350 (18-25 years) 0.1500 (26-65 years)	0.0800 (18-65 years)	-	
'LSIL'†			0.0600 (18-65 years)	
'HSIL' vs 'ICC'*	0.0300 (18-25 years) 0.1500 (26-65 years)	-	-	[37,44,49,50]
'HSIL'†	-	-	0.0800 (18-65 years)	
Death by other cause	0.0002 (15-19 years) 0.0003 (20-24 years) 0.0003 (25-29 years) 0.0005 (30-34 years) 0.0008 (35-39 years) 0.0012 (40-44 years) 0.0016 (45-49 years) 0.0024 (50-54 years) 0.0035 (55-59 years) 0.0055 (60-64 years) 0.0088 (65-69 years) 0.0161 (70-74 years)	-	-	[29]

HPV-ve', HPV-negative; 'HPV 16/18+ve', HPV-positive for genotypes 16 and 18; 'HR HPV+ve', HPV-positive for high-risk genotypes; 'LSIL', low-grade cervical lesions; 'HSIL' high-grade cervical lesions; 'ICC', cervical cancer; *, regression probability of HSIL was not considered since excisional treatment is mandatory; †, disease state of infection or SIL lesion unchanged for each Markov cycle.

Population Stratification into Disease States

Of the 2149 women studied in Chapter 3 ('Study Population'), 384 women (17.9%) were included in the 18 to 25 years age group, and the remaining 1765 women (82.1%) corresponded to the 26 to 65 years age group. Considering the 'Reference Population' (2326 women), 965 (41.5%) were included in the 18 to 25 years age group, and the remaining 1361 (58.5%) corresponded to the 26 to 65 years age group. For the 'Hypothetical Population' all women (n=2149) were aged 18 years and included in 'HPV-ve' disease state. The final stratification into disease states of the populations is presented in Table 2.

Table 2: Stratification of the two populations included in the model predictions, by disease state

	'Study Population', n (%) Age Group, years		'Reference Population', n (%) Age Group, years		'Hypothetical Population', n (%) Age Group, years
Disease State	**18-25**	**26-65**	**18-25**	**26-65**	**18-65**
'HPV-ve'	215 (56.0)	1036 (58.7)	861 (89.2)	1010 (74.2)	2149 (100)
'HPV 16/18+ve'	39 (10.2)	127 (7.2)	27 (2.8)	127 (9.3)	0 (0.0)
'HR HPV+ve'	39 (10.2)	193 (10.9)	47 (4.9)	188 (13.8)	0 (0.0)
'LSIL'	71 (18.5)	189 (10.7)	30 (3.1)	30 (2.2)	0 (0.0)
'HSIL'	20 (5.2)	201 (11.4)	0 (0.0)	6 (0.4)	0 (0.0)
'ICC'	0 (0.0)	19 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)

'HPV-ve', HPV-negative; 'HPV 16/18+ve', HPV-positive for genotypes 16 and 18; 'HR HPV+ve', HPV-positive for high-risk genotypes; 'LSIL', low-grade cervical lesions; 'HSIL', high-grade cervical lesions; 'ICC', cervical cancer.

For multiple HPV infections, whenever HPV 16 and/or 18 were detected in co-infections with other high-risk genotypes, women were included in the 'HPV 16/18+ve' disease state, because of the major contribution of these genotypes for SIL lesions development and cervical cancer.

Model predictions were calculated for two age groups (18-25 and 26-65 years), since transition probabilities varied according to age. All women were summed in relation to the year of simulation to obtain the graphic representations presented in Fig.2.

Age-specific Prevalence Curves Predicted

'Study Population'

Considering the 'HPV 16/18+ve' disease state, the highest proportion of cases would be observed within the first six years (7.8%; 166 women), and then declining over time until around 5.3% (100 women). Since women from all ages were included at the same time in the model, this peak would be linked to transient infections in younger women, as well as persistent HPV 16 and 18 infections in older women. Regarding the comparison between the different coverage rate scenarios on HPV vaccination (85% and 100%), a reduction of 32.5% would occur within the first years (5.2%; 112 women). As represented in Fig.2, both vaccination scenarios are nearly coincident, meaning that 85% of coverage rate would induce the same reduction outcome as 100% of vaccinated women aged 18 to 25 years.

The estimates obtained for 'HR HPV+ve' disease state evidenced an expected increased proportion of HR HPV-positive cases within the first two to five years, with a proportion of 9.8%

(209 women), and then declining over time until around 5.6% (105 women). The proportion of HR HPV genotypes would be maintained around 5.0% after 10 years, and it would be expected to represent persistent infections. Regarding the different scenarios for coverage rate of HPV vaccination (85% and 100%), no reduction would be expected. This scenario has probably been influenced by the initial assumption where no cross-protection was included in the simulations, inducing an unchangeable HR HPV infection estimate (regardless of the HPV vaccination scenario).

The highest proportion for low-grade cervical lesions would be registered in the first years (15.7%; 337 women), and would decrease over time until around 3.0% (58 women). Similarly to HR HPV infections, no reduction would be expected, when HPV vaccination was considered for women aged 18 to 25 years (regardless of the two coverage rates estimated). This outcome has probably been influenced by the initial stratification of women at the entry of the model, so that the expected reduction of low-grade cervical lesions, due to the decrease of HPV 16 and 18 infections, is somewhat diluted within the population's estimates. For high-grade cervical lesions, the proportion of cases would decrease within the first years to a minimal 2.8% (61 women) of cases, affecting women of all ages. When HPV vaccination was considered, again no reduction would be expected, due to the baseline stratification of women in the model. Additionally, for the estimates of cervical cancer cases, an increasing proportion of cases over time would be predictable in the 'Study Population'. This outcome results from the assumption on Markov models where the estimate of 'ICC' disease state is dependable on the prior outcome registered in the previous Markov cycle for both 'ICC' and 'HSIL' disease states. Due to ethical reasons, cervical cancer is not the endpoint on HPV vaccination clinical trial studies; thus, this increase was to be expected.

'Reference Population'

For the base-case scenario (non-vaccination), the estimates for the proportion of HPV 16 and 18 infections would reach the peak also within the first six years (7.8%; 181 women) and then slowly decreasing over time until around 4.4% (133 women). This increase proportion would be linked either to transient infections in younger women, or to persistent infections in older women. Since it was not possible to represent the simulations according to age, it is difficult to visualize an age-specific distribution of HPV-infected women. Given the initial stratification of the 'Reference Population' at the entry of the model, and as it was estimated a decrease after the initial years, it is most likely that this peak would be attributable to transient infections. If HPV vaccination was considered, a reduction of 42.7% would be observed for HPV 16 and 18 infections, when compared to the non-vaccination scenario (regardless of the coverage rate).

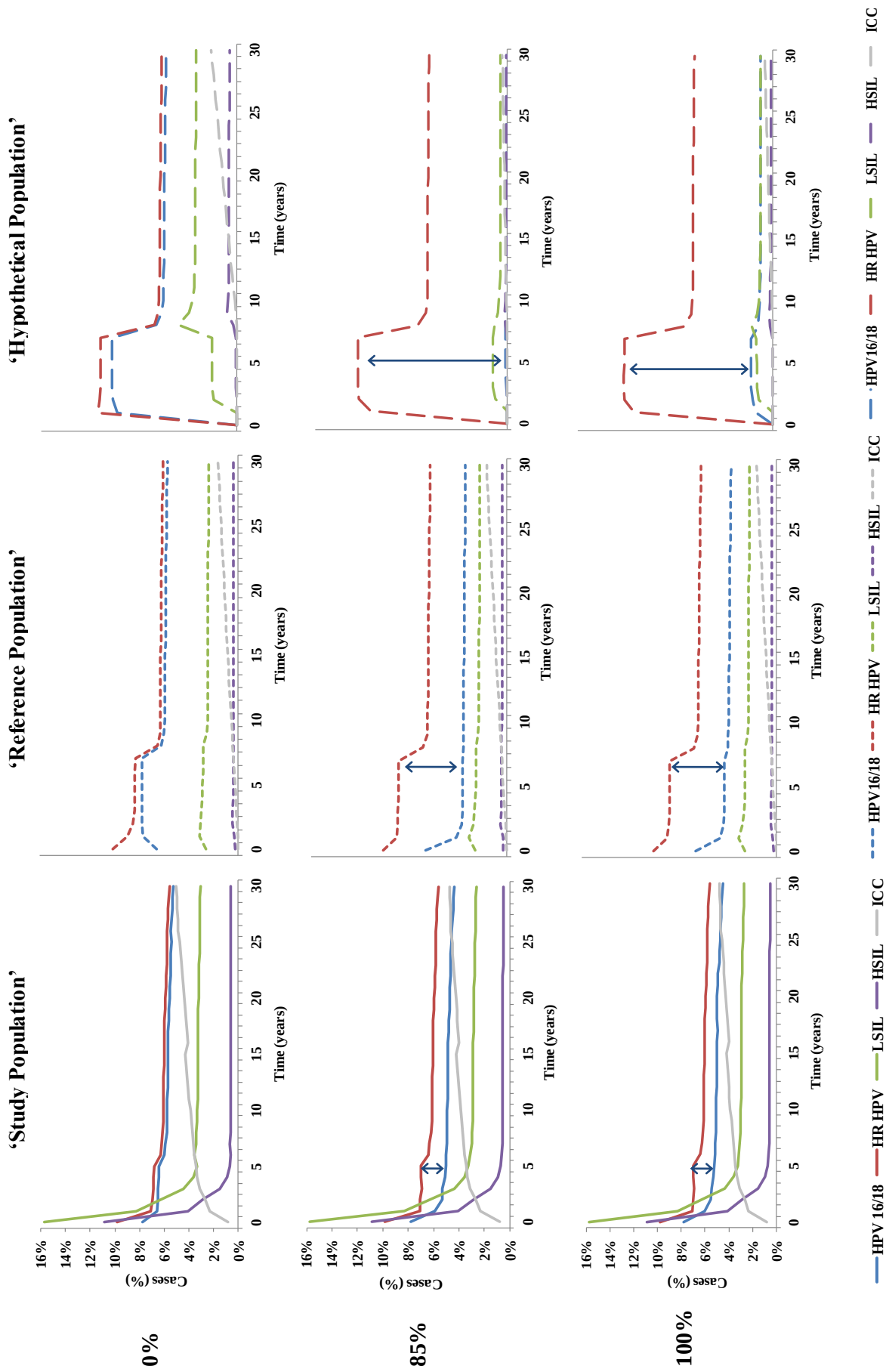

Fig.2: Representations of the different scenarios regarding HPV vaccination: 0%, 85% and 100%. 'HPV 16/18', HPV-positive for genotypes 16 and 18; 'HR HPV', HPV-positive for high-risk genotypes; 'LSIL', low-grade cervical lesions; 'HSIL', high-grade cervical lesions; 'ICC', cervical cancer. Blue arrows signal the degree of reduction observed in the progression curves.

Not Published.

The estimates of HR HPV infections in the non-vaccination scenario would reach a peak within the first six to eight years (9.0%; 235 women) and then slowly decreasing over time. Similarly to what was described for HPV 16 and 18 infections, it is more likely to be linked to transient infections, while the remainder proportion of cases after 10 years would represent persistent infections. For the comparison between vaccination scenarios, no reduction could be estimated in HR HPV infections. This outcome is also related to the absence of cross-protection influence from HPV vaccination.

The proportion of low-grade cervical lesions should remain stable over time, varying between 3.1% (60 women) and 2.2% (56 women), with no expected significant variation. This outcome would also induce a low colposcopy referral. The comparison of the HPV vaccination scenarios evidenced no estimated reduction in the proportion of cases, which could be related to the bias effect described for the 'Study Population'. Similarly, the proportion of high-grade cervical lesions would remain stable at a minimal proportion of 0.9% over time (with increasing age). This fact would be related to the low proportion of resulting high-grade cervical lesions due to the proportion estimated namely for HPV 16 and 18 infections. No reduction would be expected in the proportion of high-grade cervical lesions, if considering HPV vaccination of women aged 18 to 25 years (Fig.2). Also, cervical cancer estimates evidenced a proportion of cases of 1.6% (38 women), with no estimated impact after the comparison of HPV vaccination scenarios.

Comparison between populations

An opportunistic screening-type population would evidence an elevated proportion of HPV 16 and 18 infection cases, when compared to a routine screening-type population. For HR HPV infections, an increased proportion of 18.1% of cases would be observed for the routine screening-type population, when compared to the opportunistic screening-type population.

Regarding cervical lesions, the routine screening-type population would evidence 58.8% fewer cases of low-grade cervical lesions, with a corresponding decrease for colposcopy referral, in relation to the opportunistic screening-type population (for the first years). Differences between the two populations evidenced that the proportion of high-grade cervical lesions would remain stable at a minimal proportion of 0.9% over time for the 'Reference Population' (with increasing age). This fact would be linked to the low proportion of resulting high-grade cervical lesions due to the low proportion registered mainly for HPV 16 and 18 infections. Between the two populations, the opportunistic screening-type population would represent an increased proportion of 4.9% of cervical cancer cases, when compared to the routine screening-type population (1.6%).

Expected impact of HPV vaccination in a population not exposed to HPV infection

In opposition to the described for both populations, if HPV vaccination was to be taken by HPV-negative women (therefore not exposed to HPV infection), its expected impact would be

significantly higher. As shown in Fig.2, for HPV 16 and 18 infections, a reduction of 98.6% of cases was estimated, considering the 100% coverage rate HPV vaccination scenario. This high reduction would represent 80.8% fewer low-grade cervical lesions, 81.3% fewer high-grade cervical lesions, and ultimately, about 80.0% fewer cervical cancer cases. Regarding the differences between the two coverage rates, in the 85% HPV vaccination scenario, it was estimated a reduction of 82.3% in HPV 16 and 18 infections, 71.8% in low-grade cervical lesions, 75.0% in high-grade cervical lesions, and 66.7% in cervical cancer cases.

For the estimates of a non-vaccination scenario, HPV 16 and 18 infections would be expected to reach a proportion of 10.3% (220 women), while HR HPV infections could be estimated in 11.3% (242 women) within the first ten years. The proportions of both sub-groups of HPV-infected women would decrease to a stable proportion of 5.9% ('HPV 16/18') and 6.2% ('HR HPV'), which would be attributable to persistent infections, inducing the estimated low-grade and high-grade cervical lesions (3.4% and 0.6%, respectively). The significant decrease in the proportion of these sub-groups evidenced in Fig.2 for the 'Hypothetical Population' is consistent with the change between age groups, revealing a bias effect in the estimates.

DISCUSSION

The use of mathematical models has increased since the 1950's to assess the evolution of specific illnesses such as malaria and measles [51]. The structure of such models constitutes in the compartmentalization of the population into disease states, mutually exclusive, defined by the health state of the individuals towards the specific infection – susceptible, exposed, infected, and recovered – , while the transition between the different disease states is defined by infection, clearance, progression and regression rates. In sexually transmitted infections some questions must be addressed, such as HPV infection, like narrowing the developed model to the sexually active individuals, and involving gender separation, since the development of neoplasia is very distinct between male and female individuals.

Mathematical models can be used as tools for decision making by estimating the outcome associated with different public health-care interventions, considered as cost-effectiveness analysis when deciding a more efficient use of resources [24]. Regarding genital HPV infection, a cost-effectiveness analysis may estimate the impact of a specific intervention, v.g. HPV vaccination program, as it was aimed in the present study. The health outcomes from the model are compared to a non-vaccination scenario, providing useful data for choosing one strategy over another, and extending the knowledge from epidemiological studies to real-world situations [52,53].

For viral infections such as influenza, the vaccination impact is almost immediate and may be observed by the absence of flu in vaccinated individuals during the next cold season. Other vaccines

are administered to children to avoid serious pediatric infections, such as rubella or measles, so that the efficacy of vaccination as a preventive measure for some viral infections is observed in a short-term period. Alternatively, for HPV infection, the effect of a worldwide vaccination can hardly be observed in less than 50 to 70 years, thus, global estimates on cervical cancer reduction would only be significantly recognized by 2109 [32].

Although HPV infection has a high prevalence worldwide, the majority of infections are transient, especially in young women [3]. Being a sexually transmitted infection, it is easy to perceive that sexual behaviors are important risk factors for HPV acquisition, so that it would be meaningful to modulate the risk behaviors, v.g. sexual contacts between individuals, for HPV infection and estimate their impact on HPV prevalence. However, due to social and moral constraints this information is not available. Therefore, the presented genital HPV infection model is based on the natural history of HPV infection and cervical lesions development, in order to predict changes in HPV prevalence among Portuguese women by the introduction of HPV vaccines.

The aim was to keep the model as simple as possible without sacrificing important elements of the natural history of HPV infection. The presented model assumes an age-dependent progression and regression rates, either for HPV infection or cervical lesion cases, which is in accordance with most studies [20,24,27,49,54], and is based on the assumption that the age of HPV-infected women influence the susceptibility to acquire HPV infection, or to develop subsequent cervical lesions (by the decrease of immunological mechanisms) [55]. Estimates of progression and regression rates of HPV infection are often subjected to variability due to differences in the study design, including characteristics of the populations and HPV testing assays. This variability is also present in transition probabilities presented for SIL lesions. However, it is difficult to convert rates into probabilities that accurately reflect the natural history of HPV infection and its associated cervical lesions. Also, differences in terminology affect further research, since various studies report transitions between CIN1 to CIN3 that may be difficult to translate into LSIL and HSIL estimates [54]. The cytological SIL terminology was chosen for the assumed model because it was developed to describe differences between two different populations subjected to different health-care measures. Considering the Portuguese screening programs based on cytology testing (Chapter 2), the disease states correspondent to cervical lesions were designated into SIL lesions (LSIL and HSIL).

Question 1 "*Given the current status of the 'Study Population', the most effective approach would be to prevent HPV infection or to treat clinical symptoms?*" was addressed as a way of predicting how to proceed to reduce the burden of HPV-associated cervical lesions in the 'Study Population', which presented a high proportion of oncogenic HPV genotypes strongly associated to high-grade cervical lesions (Chapter 3). This scenario would imply a considerable use of treatment options due to the cytological characterization of the population; however, HPV vaccination could be

considered as an effective approach to women below 25 years if a significant reduction on SIL lesions could be estimated (that would result of a reduced HPV-positive cases).

A non-vaccination scenario was first estimated evidencing an evolution of the 'Study Population' over 30 years (question 1a – *"Which would be the evolution for the 'Study Population', without any clinical procedure?"*). The model has provided some insight on possible outcomes, regardless of its simplicity. Fig.2 evidences that a decrease of HPV 16 and 18 infections is to be expected, as for HR HPV infections (smaller decrease than for HPV 16 and 18). This reduction in the proportion of infection cases would represent a lower risk for the development of high-grade cervical lesions, and subsequently for the development of cervical cancer. The importance of these oncogenic genotypes in cervical cancer cases has been extensively evidenced, so that the potential decrease on HPV 16 and 18-positive cases would restrain colposcopies (and further clinical procedures) to a manageable number. In fact, it was estimated that the proportion of high-grade cervical lesions would become so low over time that they would imply fewer treatment interventions.

For HR HPV infections, all oncogenic non-vaccine genotypes were combined into a single disease state ('HR HPV+ve') because detailed information on individual genotypes is still lacking, and were further compared to an opposite disease state of HPV 16 and 18 infections, constituting two separate sub-groups for the HPV-infected state. However, this method has been shown to overestimate the infectivity of HR HPV genotypes, hence contributing for an underestimation of vaccine impact [32,56]. In fact, the comparison between both sub-groups of infection evidenced a slightly higher proportion of cases for HR HPV infection. The estimated outcomes evidenced a small decrease after the first years to a stable proportion of HR HPV infection cases around 5.0%. This proportion of cases probably represents persistent infections that would correspond to putatively low proportions of low- and high-grade cervical lesions in the 'Study Population'. Regardless of the augmented proportions determined for these women in Chapter 3, no significant improvement in the management of HPV-infected women would be needed in a near future, despite the absence of a routine screening program for these women.

Regarding cervical cancer estimates, the simulations showed an increase that would be expectable due to a mathematical artifact. In reality, less HPV-positive cases would mean less precancerous cervical lesions, and fewer cervical cancer cases. So, when estimating prevention scenarios, it is more important to observe variations for high-grade cervical lesions than for cervical cancer itself, and consequently, the projections concerning precancerous lesions may be more useful for deciding the best approach to define any clinical strategy or intervention [54,57].

Results might have been influenced by the population dimensions which are too small to induce significant variations. Moreover, the initial stratification of women at the entry of the model influenced the projections, when comparing to published data, where additional variations could be observed for the studied scenarios [20,24,45,49,54,58-61]. Various studies on mathematical modeling applied to HPV infection are based in theoretical populations involving only 'HPV-ve' individuals at

the entry of the model, and comprehending a number of women large enough (v.g. 50.000 women) to induce significant variations and reliable projections [26-29,54,57]. However, and based on epidemiological data, whenever the adequacy between the theoretical simulations and real-world populations is assured, the estimates may be assumed [19,20], as it was observed for the present study.

To address question 1b – *"To what extent HPV vaccination would contribute to the prevention of HPV-associated cervical lesions in the study population?"* – different scenarios were estimated by varying the HPV vaccination coverage rate (85% and 100%). In the first scenario the coverage rate for women aged 18 to 25 years would be the aimed by the Portuguese DGS (85%) [30], and in the second, the coverage rate for the same age group would be total (100%). These scenarios were further compared to the estimates obtained in question 1a. As evidenced by Fig.2, a reduction of 32.5% would be expected for HPV 16 and 18 infections, assuming that women aged 18 to 25 years would be vaccinated, if they were not infected by these oncogenic genotypes at baseline. No reference was made regarding which vaccine was taken, since only HPV 16 and 18 infections were modeled and both vaccines available could have been taken [55]. This estimated reduction is lower than expected, and may be linked to the initial stratification of women at the entry of the model. In fact, almost no variation was observed in the estimates of HR HPV infections, implying that even for the different scenarios of HPV vaccination, no reduction would be expected. This result is in accordance with the assumptions of the model, since no multiple infections between 'HPV 16/18' and 'HR HPV' sub-groups were allowed, and no cross-protection data was included in the design of the model. Furthermore, the 'Study Population' was constituted by a relatively low number of women (N=2149), which restricts the estimates of the model in fewer variations and lower reduction outcomes, as described by others [26,45,54]. Nonetheless, an interesting outcome was obtained, which was the almost coincident estimates for both 85% and 100% HPV vaccination coverage rates, which brings up question 1c – *"How does HPV vaccination impact on the prevention of HPV 16 and 18 infections in the 'Study Population' for a coverage rate of 85%?"*. This means that the 85% coverage rate proposed by the Portuguese DGS would induce the same prevention outcome than the 100% coverage rate. This result may also evidence some degree of herd immunity in the female population, which is desirable to achieve better results [55,62].

To address question 2 – *"For each disease state what would be the main differences state between routine screening-based and opportunistic screening-based populations?"* – the estimated scenarios between the 'Study Population' and the 'Reference Population' were compared. First, on *"How would the routine screening impact on HPV infection dynamics for a 'Reference Population'?"* (question 2a), the estimates for a routine screening-based population were analyzed. The proportion of HPV 16 and 18 positive cases was estimated to increase in the first years and then decrease to a constant proportion of less than 5.0%. The initial increase would represent transient infections, while the remaining 5.0% would probably represent persistent infections affecting older

women, and requiring follow-up either by cytology, colposcopy, HPV testing or specific molecular markers. For HR HPV infections, a high proportion would be maintained during the first years and then slowly decreasing. Again, the remaining proportion of HR HPV infection cases over time would represent persistent infections with a putative impact on the development of cervical lesions.

For the 'Reference Population', only the constant proportion of HPV-positive cases during the whole modeling period (30 years), would represent an increased risk for the development of cervical lesions. The estimates point to a low proportion of low-grade cervical lesions, and even a lower proportion of high-grade cervical lesions. Consequently, this routine screening-based population would evidence a lower proportion of cervical cancer cases, which is in accordance with the expected prevention provided by a screening program [20,24,55]. Furthermore, for question 2b – *"Different coverage rates (85%* versus *100%) of HPV vaccination would impact on the 'Reference Population'?"* – it was evidenced that different coverage rates would only induce a significant decrease of HPV 16 and 18 infections, which has been proposed [20] to reduce the management needed in a routine screening context. Nonetheless, it was shown that the 'Study Population' would evidence a small increase in the proportion of cervical lesions, when compared to the 'Reference Population', demonstrating the influence of routine screening in preventing cervical lesions associated to HPV infections. Cervical cancer screening programs, even in a co-approach including HPV vaccination, would induce a significant decrease to every disease state of the model [24,49,54,58,63-65], and more importantly, this impact was evidenced by the expected decrease in cervical cancer cases in the 'Reference Population', when compared to the 'Study Population'.

Finally, to address question 3 – *"How would be HPV vaccination effective in a population not exposed to HPV infection?"* – additional scenarios were estimated in an 'Hypothetical Population'. This population consisted in 2149 women that were all included in the 'HPV-ve' disease state at the entry of the model, as assumed by the modeler, to enable further comparisons. All women were assumed to have 18 years (youngest age for entering the model in the 'Study Population'). The different scenarios evidenced a significant prevention of HPV 16 and 18 infections that would represent a significant reduction of low- and high-grade cervical lesions. Also, less cervical cancer cases would be expected among the 'Hypothetical Population', when compared to the other populations. Therefore, as extrapolated in other studies [20,26,28,29,60], when HPV vaccination is administered to a female population not previously exposed to HPV, it is highly effective in reducing HPV-associated cervical lesions development. This outcome provided by various mathematical models [26,54,60,61] justified the recommendation of HPV vaccination for adolescent girls, considering that the age for sexual debut is reducing, shifting the peak of HPV infections to younger women than what was previously observed [34,43].

In conclusion, through the analysis and comparison of every estimate and scenario, in addition to epidemiological data from Chapters 3 to 8, it was possible to gain further understanding

and insight of the special characteristics of the 'Study Population'. The decision underlying the mathematical model assumed in this study will favor a clinical approach, where HPV-infected women would be followed through HPV testing and cytology, and in some cases, colposcopy and/or molecular markers. Furthermore, recently published studies [32,66] assume that a significant decrease on cervical cancer incidence rates will only be observed many years from now, considering that the majority of women has been already exposed and (if sexually active) remains at risk of being infected by HPV. Therefore, and regarding cervical cancer prevention, HPV vaccination will have a long-term impact, regardless of its highly significant recommendation to adolescent girls [32,66]. As referred elsewhere, if one woman chooses to be vaccinated, it is unlikely to induce changes in cervical cancer incidence rates [66].

This study may be assumed as limitative in the conclusions, since the described health outcomes can only be applied to the studied populations. In the absence of accurate epidemiological parameters, as there are no studies involving Portuguese populations to determine natural transitions between stages of HPV infection, future application of dynamic mathematical models will surely contribute more extensively to inform health authorities on better strategies for reducing cervical cancer. Especially when considering the tendency regarding the positive input pressure that HPV vaccination may represent for Portuguese women, an estimated reduction of HPV 16 and 18 infections was evidenced by the present study.

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CHAPTER 10

GLOBAL ANALYSIS:
TOWARDS CERVICAL CANCER PREVENTION STRATEGIES

So far, the research on human papillomavirus (HPV) has gained tremendous knowledge and insight about HPV infectious process and its associated diseases; has formulated prevention strategies for one of the most frequent sexually transmitted infections (STI) in the world; has developed significantly sensitive molecular diagnostic assays for early detection of the viral DNA; and has developed prophylactic vaccines evidencing encouraging efficacy results. In the course of progressing this far on behalf of cervical cancer prevention, as one of the cancers that affects more women at ages 15 to 44 years, HPV research encountered more challenges to overcome, especially social and economic, to bring new options on short term management of HPV-infected women, and on an aimed long-term viral elimination. Below, the major aspects presented in this PhD thesis are discussed, and future perspectives in HPV research are addressed, in view of incrementing cervical cancer prevention, in particular among Portuguese women.

Cervical cancer is one of the major malignancies affecting women worldwide. In an era where vaccination has become a reality for the prevention of some genotypes, the paradigm of HPV infection is shifting. In the words of the Nobel Prize winner Harold zur Hausen, "*instead of pursuing to avoid HPV infection, or cervical cancer development mechanisms, the path to follow is prevention*" (TV interview, October 2013).

Main Findings

In Portugal, cervical cancer incidence rate is high (12.2 per 100,000 women), highlighting the importance of continuously monitor HPV-infected women [7,13]. Epidemiologic data is always required, including in populations at increased risk, as the women evaluated through the present PhD thesis. High-risk HPV infection was detected frequently in normal cytology cases (22.6%), which can constitute a risk factor for cervical cancer development, despite the potential bias over HPV positivity rate considering that the study was based on an opportunistic screening-type population (Chapter 3). In fact, the characteristics of the Study Population may have increased and overstated HPV prevalence, consequently increasing its proportion among cervical disease. Moreover, women included in this study were referred to HPV testing due to the identification of cytological abnormalities, which boosts the probability of HPV-positive results. In addition, many women were undergoing HPV testing for the first time, increasing the possibility of identifying transient infections, especially in young women. This raises the question on how to manage HPV infection in younger women, as an elevated HPV positivity rate was determined in women below 30 years (peak registered in women aged 25 to 29 years).

Regarding HPV RNA testing, the positivity rate in women with normal cytology was only 6.0% in women below 30 years of age, which contrasted significantly with the elevated percentage of HPV DNA detected in younger women (28.6%). For women above 30 years, HPV RNA detection rate decreased to 4.9%, evidencing the fewer clinically active HPV infections due to viral persistency, and therefore, at higher risk for cervical lesion development [280].

The genotype-specific profile that was identified, demonstrated that HPV 16, 31, 53, 51, 18 and 66 were the most frequent genotypes. The prevalence rates of these genotypes differed according to cytological diagnosis evidencing a significant association of HPV 16 and 18 to cervical cancer development. These two oncogenic genotypes were responsible for more than 65.0% of high-grade cervical lesions, meaning that HPV vaccines currently available in Portugal (which include these genotypes) may contribute to the decrease of HPV 16/18-associated cervical diseases in our country. It is important to continuously monitor HPV dissemination, especially in populations with high disease rate in order to discriminate the main circulating HPV genotypes and their specific contribution to high-grade cervical lesions, putatively enabling statistical validations with fewer women [228,229,235]. Moreover, the vast majority of Portuguese women have already been exposed to HPV infection prior to vaccination implementation, turning HPV genotype-specific distribution evaluation as particularly important to clarify future public health decisions [151,281,282].

Chapters 4 to 6 evidenced the importance of HPV testing in cervical cancer screening strategies, as opposed to the routinely applied cytology. HPV testing has the advantage to increase sensitivity of viral detection, since this is one of cytology's disadvantages. Routine cytology testing provides cervical disease detection demonstrating high specificity, while complementary HPV testing can improve early detection of viral infection due to higher sensitivity. However, in younger women, HPV testing will over-estimate the number of false positive results (v.g. high-risk HPV positivity without subsequent high-grade cervical lesions development). In opposition, for older women, HPV testing prior to cytology represents a better strategy due to its higher sensitivity which is most linked to persistent infections [228-230,238,282-287]. Moreover, the high proportion of oncogenic HPV genotypes circulating in Portugal is of some concern, due to their higher probability of inducing persistent infections and subsequent cervical disease development.

The primary testing in cervical cancer screening is of some importance since the loss of specificity would promote unnecessary treatment procedures with associated costs. It has been shown that HPV RNA testing increases the specificity of cervical disease detection, without putting at risk the increased sensitivity of HPV DNA testing. Thus, HPV RNA testing could constitute an alternative for the management of HPV-infected women, while avoiding potentially unnecessary colposcopy referral [238,280,281].

HPV 16 and 18 have a well established significant association to cervical cancer development [13,151,160]. Considering this, in Chapters 7 and 8, it was exploited the prognostic significance of HPV 16 and 18 loads, of viral physical status, and the predictive value of these potential molecular markers. This fact raises the possibility of additional screening stages in order to better discriminate HPV infections that would develop more rapidly in cervical cancer cases.

An inverse relation between the two molecular markers, within the same genotype, could be observed. A significant association between high HPV 16 viral load and high-grade cervical lesions development was evidenced; in opposition, HPV 16 DNA physical status was not associated with cervical lesion development, for the same sample. In fact, the HPV 16 viral load increased significantly with the severity of clinical diagnosis, while the DNA determined in the linear form (with integration potential) was not often detected, even in high-grade cervical lesions. This pattern suggests the need for closely monitor the quantitation of viral load in women infected by this genotype, while HPV 16 integration seems not to be essential for malignant transformation, since concomitant forms were the most frequently detected, independently of the clinical diagnosis [270,288,289].

Regarding HPV 18-positive cases, viral load only evidenced a possible association with the persistency of HPV infection, while a significant association was evidenced between the presence of HPV 18 linear forms and cervical disease progression. The higher values of HPV 18 viral load in low-grade cervical lesions could favor an increased rate of HPV 18 integration, as linear forms identification increased with cervical disease diagnosis. Furthermore, when integrated HPV 18 was

detected, a decrease in the correspondent viral load was identified, which could be expected considering that after viral genome integration, viral replication stops. At this stage, viral replication depends of host cell mechanisms, since viral genome becomes part of host genome [290-292]. Consequently, during the final stages of cervical carcinogenesis, HPV 18 viral load would not influence progression to cervical cancer, but the determination of HPV 18 DNA physical status would be predictive of the risk of malignant transformation [276]. These molecular markers may be included in routine cervical cancer screening algorithms, and may act as effective triage tools for risk stratification of HPV 16 and 18-infected women, potentially reducing the need for colposcopy and biopsy referral, which are uncomfortable, expensive and time consuming invasive procedures [293,294]. Furthermore, HPV 18 has the predisposition to infect glandular cells, where adenocarcinomas are particularly difficult to detect cytologically; thus, viral load and physical status are of additional significance in women infected with HPV 18, when compared to HPV 16. Nevertheless, various measurements are required over time, to ascertain a persistent infection and an accurate risk assessment through these molecular markers.

Chapter 2 addressed the scenario of cervical cancer screening measures in Portugal, where screening programs are regionally-based, implying disparities on health-care policies among regions. These measures may be further weakened if the screening programs are not monitored to evaluate its success. At present, there are a few gaps that affect the prevention provided by the implemented screening programs. These gaps are related to the effectiveness in recruiting eligible women for screening, and to differences on primary testing and age range of screened women. This fact raises the need to apply additional measures, which may adequate more properly the treatment options to those lesions that constitute a real increased risk for high-grade cervical lesions development. To assure an increased specificity in specific stages of the screening algorithm is a way to detect cervical disease more effectively [236,295-297], while to propose alternative algorithms may improve management of HPV-infected women (Fig.1).

Additionally, in Chapter 9 it was described how mathematical models would help to better decide between clinical strategies by estimating different scenarios for HPV natural history. Here, it was chosen to develop a simple compartmental Markov model that would describe the evolution of the studied population, and it was further exploited the prevention induced by the introduction of HPV vaccines, measured in the proportion of reduced cases of HPV infection and cervical disease. It was estimated that high-risk HPV infection, namely HPV 16 and 18, would remain high within the first years, and then decreasing over time, thus representing a low proportion of high-grade cervical lesions. This means no expected increase of clinical resources would be necessary for the management and treatment of HPV-infected women. Moreover, the comparison between the ‘Study Population’ and a ‘Reference Population’ evidenced the importance of routine screening for cervical cancer prevention. Despite the aimed benefits in reducing HPV cervical disease as a result of the current vaccination programs, successful HPV management should not rely on primary prevention

only, but also on early detection and treatment of cervical lesions, in order to achieve more immediate effects.

All the chapters contributed to some extend for a formulated strategy (Fig.1) that would provide a better management of HPV-infected women in Portugal: i) Chapter 3 have provided baseline information on HPV genotype-specific distribution and its associated cervical lesions; ii) Chapters 4 to 6 provided clinical relevance to the use of HPV DNA and HPV RNA testing; iii) Chapters 7 and 8 provided clinical significance on two important and emerging molecular markers; iv) Chapter 2 provided the actual scenario on screening strategies that would need improvement; and v) Chapter 9 provided future possible scenarios for the Study Population.

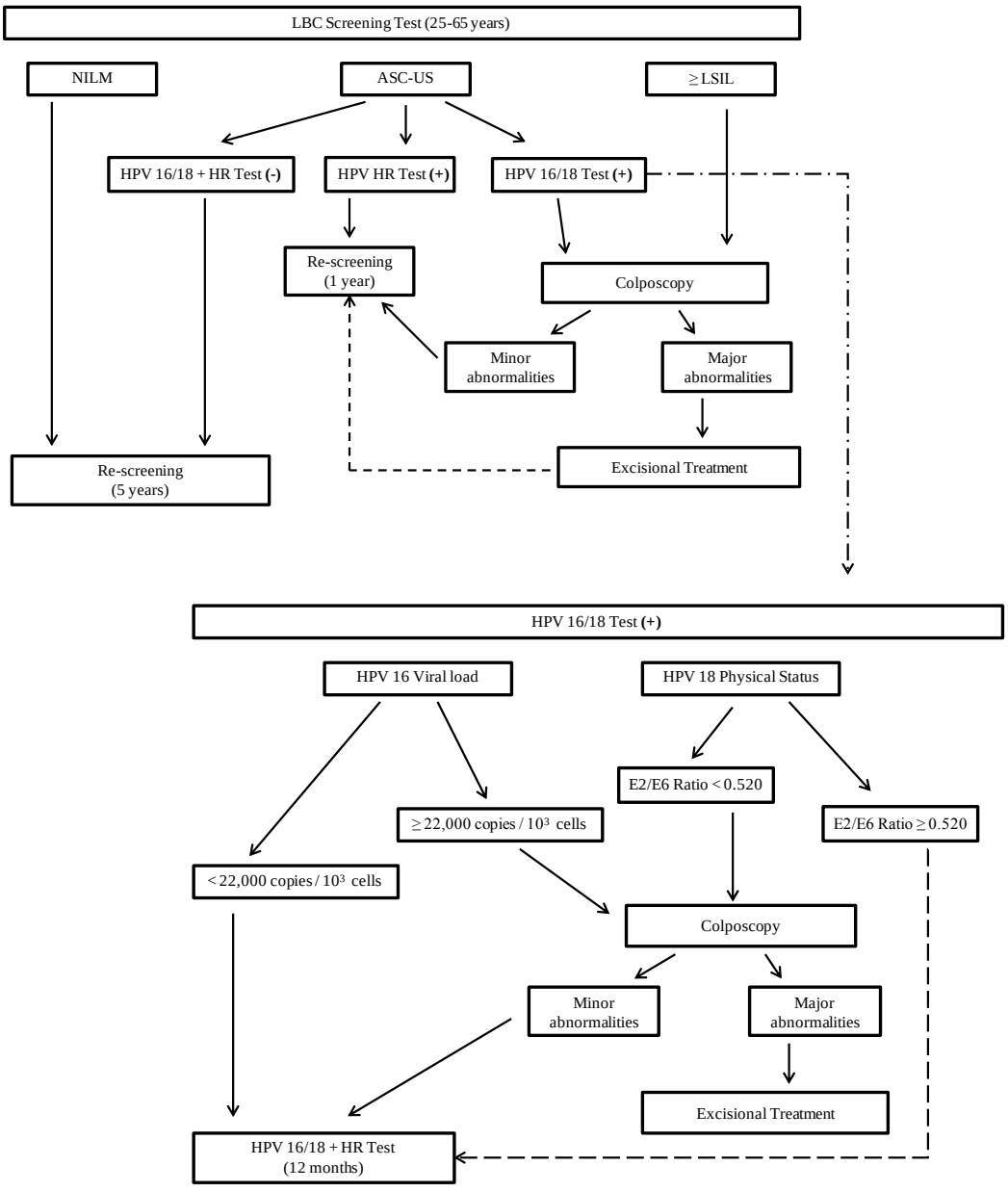

Fig.1: Algorithm proposal for HPV/cervical cancer routine screening.

Final Remarks

HPV infection is so prevalent that is almost unavoidable, affecting millions of women around the world, including Portugal. Thus, it is urgent to rationalize better health-care and a higher quality of life for women, and also diffuse the right message to the society, while taking an active part on preventing the dissemination of this STI, which begins by a safer behavioral attitude.

Large HPV longitudinal studies, either prospective or retrospective, as well as HPV screening evaluations will provide data that will inform about prevention programs adequate to each specific setting (country, region, age, sexual habits, risk factors, etc). Women exposed to HPV and potentially harboring persistent infections, constitute cervical cancer candidates, and should beneficiate from accurate and up-to-date diagnostic tests and treatment options, being surgery the least desirable compared to early and regular screening.

HPV infection is demanding in so many aspects, making prevention strategies even more challenging. To globally embrace a definitive reduction on cervical cancer incidence, it is important to highlight the special features intrinsic of each genotype's natural history of carcinogenesis. In this context, basic research on HPV viral mechanisms responsible for the development of anogenital cancers remains essential. These studies should be conducted for each genotype, after establishment of its specific epidemiological contribution. This way, underlying viral mechanisms that resulted in successful adaptations to host cell environment should be further investigated. One example is the more aggressive behavior of HPV 18 in cervical cancer development. Why is HPV 18 more prone to infect glandular cells and induce the development of adenocarcinomas with worse prognosis? What nucleic acid mutations and variations are responsible for a more rapid progression to cervical cancer? What differences result in an increased oncogenic role for viral proteins? These questions have now the possibility to get answered through new-generation sequencing. This new technology will provide important information on viral genomic rearrangements and valuable insight on viral full-genome data that still remain unknown for most genotypes. High quality sequences obtained from full genome sequencing offer new possibilities and novel information to: 1) characterize genomic viral variants; 2) assess mechanisms of oncogenicity for these variants; 3) identify genomic recombination sequences from multiple HPV infections; 4) establish specific routes used from molecular variants within host cell environment; 5) identify novel sequences with malignant transformation potential; and 6) identify novel genotypes [298].

There are many other fields to pursue on HPV research with new-generation sequencing: What are the most common viral integration sites on host cell genome? What determines the viral

disruption site and the consequent integration site? What host cell mechanisms are affected by viral integration? What integration sites represent an advantage to a more aggressive behavior? And if so, how viral mechanisms act to achieve this advantage? Which viral proteins take a role in this process, and which nucleic acid variations are more successful? These questions would provide interesting data on HPV research and cervical cancer prevention. The progress in preventing HPV-related diseases becomes attainable if studies are conducted throughout all stages of infection and disease, improving measures and technology, and translating the obtained results into the clinical field [299,300].

On a screening context, as a result of substantial population growth in developed and developing areas, universal screening and effective early treatment are issues that have to be readily addressed. Additionally, it would be important to identify potential target DNA sequences of diagnostic interest using new-generation sequencing. These sequences could be further used for the improvement of current HPV tests, as well as for the development of new tests that would bring advances in HPV detection and risk assessment. In fact, a more effective prevention may be linked to specific strategies at a genotype level. For instance, it is still unknown if other high-risk genotypes will present a higher contribution to cervical cancer development, in particular after the expected decrease of HPV 16 and 18 prevalence through vaccination. One statement consists in the assumption that some degree of cross-protection conferred by HPV vaccination will prevent genotype replacement in cervical cancer incidence [301]. Nonetheless, it is likely to observe some increase of otehr high-risk HPV genotypes in association with precancerous cervical lesions, which might have to be included in future HPV vaccines [213,221,302-304].

Concerning the immunology related to HPV infection, it would be important to clarify which viral mutations may contribute to elude the host immune system, as a way to “silence” HPV persistent infection without recognition from host cells. Recently, the recurrence to adjuvants to boost T-cell mediate response from the host immune system has evidenced interesting results [305]. Moreover, the arising of mutations on HPV 16 and 18 variants may contribute to escape the immunity conferred by HPV vaccines and, in this case, it will be crucial to identify these mutations [298]. Ultimately, it will be fundamental to establish a match between viral features and host immune responses to better understand viral-host interactions, as recently published [306].

HPV is a virus well adapted to infect human epithelial cells, and have co-evolved within the host, raising questions on how the virus manages to remain so widespread in the population, even increasing its incidence in some anogenital cancers. Such questions remain to be answered and require extended research. Furthermore, two of the most oncogenic viral proteins, E6 and E7, constitute potential targets for research namely to the understanding of how these oncoproteins

interact with cellular proteins p53 and pRb that was shown to conduct to the malignant transformation of host cells, or identifying particular features linked to their oncogenicity (at a genotype level) [296,298-300]. Additionally, specific mechanisms that are responsible for differences at a protein level remain unknown, and will most certainly influence the progression to cervical cancer.

The work presented through the present PhD thesis exposes a comprehensive and extensive research on HPV that ultimately may result in prevention strategies more appropriate to Portuguese women. The identification of some gaps in the actual HPV screening programs implemented in Portugal enables further improvement of available technologies rationalizing the use of resources more adequate and applicable, while providing enhanced health-care to Portuguese women.

CHAPTER 11

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