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Characterization of High-Risk Human Papillomavirus Multiple Infections in Cervical Cancer Screening and Correlation with Vaginal Microbiome

Andreia Filipa da Silva Rosário

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CHARACTERIZATION OF HIGH-RISK HUMAN PAPILLOMAVIRUS MULTIPLE INFECTIONS IN CERVICAL CANCER SCREENING AND CORRELATION WITH VAGINAL MICROBIOME

MSc Dissertation in Medicine and Molecular Oncology submitted to the FACULTY OF MEDICINE, UNIVERSITY OF PORTO

Andreia Filipa da Silva Rosário, BSc
Molecular Oncology & Viral Pathology Group, IPO-Porto Research Center (CI-IPOP), IPO Porto FG - EPE, Porto, Portugal
Clinical Pathology Service, Clinical and Medical Pathology Department, IPO Porto FG - EPE, Porto, Portugal

SUPERVISION
Hugo Manuel Lopes de Sousa, Professor, PhD
Molecular Oncology & Viral Pathology Group, IPO-Porto Research Center (CI-IPOP), IPO Porto FG - EPE, Porto, Portugal
Clinical Pathology Service, Clinical and Medical Pathology Department, IPO Porto FG - EPE, Porto, Portugal

CO-SUPERVISION
Rui Manuel de Medeiros Melo Silva, Invited Professor, PharmD, PhD
Molecular Oncology & Viral Pathology Group, IPO-Porto Research Center (CI-IPOP), IPO Porto FG - EPE, Porto, Portugal
Clinical Pathology Service, Clinical and Medical Pathology Department, IPO Porto FG - EPE, Porto, Portugal

Ana da Conceição Saraiva e Sousa, MSc
Molecular Oncology & Viral Pathology Group, IPO-Porto Research Center (CI-IPOP), IPO Porto FG - EPE, Porto, Portugal

Pathological Anatomy Service, Clinical and Medical Pathology Department, IPO Porto FG - EPE, Porto, Portugal

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"Try one, two, three times and if possible try the fourth, fifth, and as many times as necessary. Just do not give up on the first time attempts, persistence is a friend of conquest. If you want to get where most do not go, do what most do not."

Bill Gates

E assim termino mais um capítulo importante da minha vida académica e pessoal, capítulo este que não seria possível sem o contributo de várias pessoas que assumiram um papel importante. Por isso, agradeço a todos aqueles que contribuíram para esta etapa.

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Obrigada!

ABBREVIATIONS LIST

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A
ACES – Agrupamento de Centros de Saúde
AGC – Atypical Glandular Cells
ARS – Administração Regional de Saúde
ASC-H – Atypical Squamous Cells High-Grade
ASC-US – Atypical Squamous Cells of Undetermined Significance

C
CSTs – Community State Types

D
DNA – Deoxyribonucleic Acid

I
ICC – Invasive Cervical Cancer

H
HSIL – High Grade Epithelial Lesion
HPV – Human Papillomavirus
Hr-HPV – High-Risk Human Papillomavirus

L
LSIL – Low Grade Epithelial Lesion

P
PVs – Papillomaviruses

R
RCCU – Cervical Cancer Screening

W
WHO – World Health Organization

OUTLINE OF THE THESIS

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In **Chapter I**, the rational for this study will be presented focusing Human Papillomavirus (HPV) and cervical cancer association and carcinogenesis. Then, epidemiology, prevention (primary and secondary) and different genotypes of HPV. The last topic is microbiome and correlation with cytological diagnosis. Additionally, the aims of this thesis will also be described in this chapter.

In **Chapter II**, it will be presented an article which will briefly explain the impact of High-risk HPV (Hr-HPV) in cervical disease in the screening program from northern region of Portugal. The chapter is presented as the following article:

Rosário A, Sousa A, Marinho-Dias J, Medeiros R, Lobo C, Leça L, Tavares F, Martins G, Monteiro P, Henrique R, Sousa H. Impact of High-Risk Human Papillomavirus Genotyping in Cervical Disease in the Northern Region of Portugal: Real World Data from Regional Cervical Cancer Screening Program. (submitted).

In **Chapter III**, it will be characterized the vaginal microbiome and their correlation with cytological diagnosis and Hr-HPV genotype in cervical disease in the screening program from northern region of Portugal. The chapter is presented as the following article:

Rosário A, Sousa A, Varandas T, Marinho-Dias J, Medeiros R, Martins G, Monteiro P, Sousa H. Characterization of Vaginal Microbiome in Cervical Samples from the Regional Cervical Cancer Screening Program of the Northern Region of Portugal. (submitted).

In **Chapter IV**, it will be discussed the results obtained in this thesis.

In **Chapter V**, it will be summarized the conclusions of this thesis and additionally, it will be suggested future work in this theme.

In **Chapter VI**, it will be presented the references of the thesis.

Addicionally, part of the results of this work were presented in oral communication at IJUP2022 - Young Research Meeting (IJUP'22 – 15.º Encontro de Investigação Jovem da Universidade do Porto) and accepted for poster presentation at the ESCV22 (European Society of Clinical Virology) and at the 5th National Meeting of Young Researchers in Oncology (5.º ENJIO – 5.º Encontro Nacional de Jovens Investigadores em Oncologia) 2022.

INDEX

INDEX

ABSTRACT

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Introduction: Worldwide in 2020, Cervical Cancer was the fourth most frequently diagnosed cancer and the fourth main cause of cancer death in women and Portugal, in 2020 it was estimated a total of 865 new cases and 379 deaths. The cervical cancer screening in the Northern Region of Portugal using full Hr-HPV genotyping as the primary approach for cervical cancer screening was implemented in 2016 for women aged 25–60 years old. Here we explore the correlation between Hr-HPV genotypes, cytology and Vaginal Microbiome to improve the knowledge on cervical disease in Portugal.

Material and Methods: We have performed a retrospective analysis of data collected from women who participated in the Regional Cervical Cancer Screening Program from the Northern Region of Portugal (RCCU) between 2016 to 2021. Furthermore, we have selected a cohort of women from RCCU collected between September 2021 and November 2021 with Hr-HPV positive result to study the Vaginal Microbiome and its impact on cervical disease.

Results: Data from RCCU between 2016 to 2021 revealed a prevalence of Hr-HPV of 12.50% in a total of 462,401 women screened. Results showed that multiple infections with two or more Hr-HPVs represent 27.14% of all cases; the most common Hr-HPVs found were 68, 31, 51, 16, 39, 66 and 56; and Hr-HPV included in the 9-valent vaccine (16, 18, 31, 33, 45, 52 and 58) represent 55.37% of all positive cases. Cytological results for Hr-HPV positive women (n=57.103) revealed that 65.68% have NILM, 20.83% have ASC-US, 8.86% have LSIL, 1.65% have HSIL, 2.85% have ASC-H, 0.09% have AGC, and 9 women with AdC and 10 with ICC. The Hr-HPVs included in 9-valent vaccine are associated with 52.13%% of NILM, 59.21.% of ASC-US, 55.06% of LSIL, 90.14%% of HSIL, 83.50% of ASC-H, 83.02% of AGC, 77.78% of AdC and 100% of ICC.

For the microbiome analysis we enrolled a total of 807 Hr-HPV positive women with cytologies: (39.5%) NILM, 111 (13.8%) ASC-US, 114 (14.1%) ASC-H, 215 (26.6%) LSIL, 47 (5.8%) HSIL and 1 (0.1%) AdC. Overall, we found a high prevalence of infectionsbeing the most frequent ones *U. parvum* (52.5%), *Gardnerella vaginalis* (34.5%), *Atopobium vaginae* (32.6%), *Lactobacillus* spp. (30.7%) and *M. hominis* (23.5%). The risk analysis regarding cytology revealed that infection by *Megasphaera* Tipo 1, *Lactobacillus* spp. , *Gardnerella vaginalis*, bacteria associated to bacterial vaginosis (BVAB2), *Atopobium vaginae* and *Mobiluncus* spp. are associated with decreased risk for the development of any cervical abnormality ($p<0.001$); infection by *U. urealyticum* and *M. hominis* are associated with increased risk of multiple Hr-HPV infections; infection by *Lactobacillus* spp., *Gardnerella vaginalis* (GV) are associated with decreased risk of either HPV-16/18 or HPV-9val genotypes while infection M *hominis* and *Mobiluncus* spp. are associated with increased risk of infection by one HPV-9val genotypes.

Conclusions: This study shows that HPV prevalence in 25-64 years old is 12% and that the Hr-HPVs included in the 9-valent vaccine account for a high percentage of cases with cytological abnormalities, being HPV-16, -18, -31 and -33 the most associated with >HSIL lesions. These data showed that overall *Lactobacillus* spp. and *Gardnerella vaginalis* are associated with a protective effect for either HPV-infection of cervical abnormality development while *U. urealyticum*, *M. hominis and Mobiluncus* spp. are more frequently associated with increased risk. This study provides important data to be used in the clinical approach and management of Hr-HPV positive women.

Key-words: Human Papillomavirus, cervical cancer, Regional Cervical Cancer Screening Program, Hr-HPV, genotypes. Vaginal microbiome

RESUMO

RESUMO

Introdução: A nível mundial em 2020, o Cancro do Colo do Útero foi o quarto cancro mais frequentemente diagnosticado e a quarta principal causa de morte por cancro nas mulheres. Em Portugal, em 2020 estimou-se um total de 865 novos casos e 379 mortes. O rastreio do Cancro do Colo do Útero na Região Norte de Portugal, utilizando a genotipagem completa do Hr-HPV como abordagem primária para o rastreio do Cancro do Colo do Útero, foi implementado em 2016 em mulheres com idades compreendidas entre os 25-60 anos. A correlação entre genótipos de Hr-HPV, citologia e microbioma vaginal servirá para melhorar o conhecimento sobre o cancro do colo do útero em Portugal.

Material e Métodos: Realizámos uma análise retrospetiva dos dados recolhidos de mulheres que participaram no Programa Regional de Rastreio do Cancro do Colo do Útero da Região Norte de Portugal (RCCU) entre 2016 e 2021. Além disso, selecionámos uma coorte de mulheres do RCCU recolhidas entre Setembro de 2021 e Novembro de 2021 com resultado positivo do Hr-HPV para estudar o microbioma vaginal e o seu impacto no cancro do colo do útero.

Resultados: Os dados do RCCU entre 2016 e 2021 revelaram uma prevalência de Hr-HPV de 12,50% num total de 462.401 mulheres rastreadas. Os resultados mostraram que as infeções múltiplas com dois ou mais Hr-HPV representam 27,14% de todos os casos; os Hr-HPV mais comuns foram -68, -31, -51, -16, -39, -66 e -56; os Hr-HPV incluídos na vacina 9-valente (-16, -18, -31, -33, -45, -52 e -58) representam 55,37% de todos os casos positivos. Os resultados citológicos em mulheres positivas para Hr-HPV (n=57.103) revelaram que 65,68% têm NILM, 20,83% têm ASC-US, 8,86% têm LSIL, 1,65% têm HSIL, 2,85% têm ASC-H, 0,09% têm AGC, 9 mulheres com AdC e 10 com ICC. Os Hr-HPVs incluídos na vacina 9-valente estão associados a 52,13% de NILM, 59,21% de ASC-US, 55,06% de LSIL, 90,14% de HSIL, 83,50% de ASC-H, 83,02% de AGC, 77,78% de AdC e 100% de ICC.

Para a análise microbiológica utilizámos um total de 807 mulheres Hr-HPV positivas com citologias: (39,5%) NILM, 111 (13,8%) ASC-US, 114 (14,1%) ASC-H, 215 (26,6%) LSIL, 47 (5,8%) HSIL e 1 (0,1%) AdC. Globalmente, encontrámos uma alta prevalência de infeções sendo as mais frequentes *U. parvum* (52,5%), *Gardnerella vaginalis* (34,5%), *Atopobium vaginae* (32,6%), *Lactobacillus spp*. (30,7%) e *M. hominis* (23,5%). A análise de risco relativa à citologia revelou que a infeção por *Megasphaera* Tipo 1, *Lactobacillus spp*., *Gardnerella vaginalis*, bactérias associadas à vaginose bacteriana (BVAB2), *Atopobium vaginae* e *Mobiluncus spp*. estão associadas à diminuição do risco de desenvolvimento de qualquer anomalia cervical ($p<0,001$); infeção por *U. urealyticum* e *M. hominis* estão associadas ao aumento do risco de infeções múltiplas por Hr-HPV;

infeções por *Lactobacillus spp* e *Gardnerella vaginalis* (GV) estão associadas a uma diminuição do risco de HPV-16/18 ou genótipos HPV-9val, enquanto as infeções por *M. hominis* e *Mobiluncus spp*. estão associadas a um aumento do risco de infeção por um genótipo HPV-9val.

Conclusões: Este estudo mostra que a prevalência do HPV entre os 25 e os 64 anos é de 12% e que os Hr-HPVs incluídos na vacina 9-valente são responsáveis por uma elevada percentagem de casos com anomalias citológicas, sendo o HPV-16, -18, -31 e -33 os mais associados a lesões >HSIL. Estes dados mostraram que o *Lactobacillus spp*. e *Gardnerella vaginalis* em geral estão associados a um efeito protetor quer para a infeção por HPV quer do desenvolvimento de anomalias cervicais, enquanto *U. urealyticum, M. hominis* e *Mobiluncus spp*. estão mais frequentemente associados a um risco acrescido. Este estudo fornece dados importantes a serem utilizados na abordagem e gestão clínica das mulheres positivas para Hr-HPV.

Palavras-chave: Vírus do Papiloma Humano, cancro cervical, Programa Regional de Rastreio do Cancro do Colo do Útero, Hr-HPV, genótipos, Microbioma vaginal.

CHAPTER I

BACKGROUND

1. CANCER

According to the World Health Organization (WHO), cancer is “a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can invade adjoining parts of the body and/or spread to other organs” [1]. There are more than 100 different types of cancer, since it can affect practically every part of the body and can have many anatomic and molecular subtypes [1,2].

In Portugal, from 2020 to 2040 it is estimated nearly a 17% increment in the number of new cancer cases [3]. Additionally, the number of deaths attributable to cancer in the Portuguese population is expected to increased approximately 27% in the next two decades, with a higher increment in men than in women [3,4] – **Figure 1**.

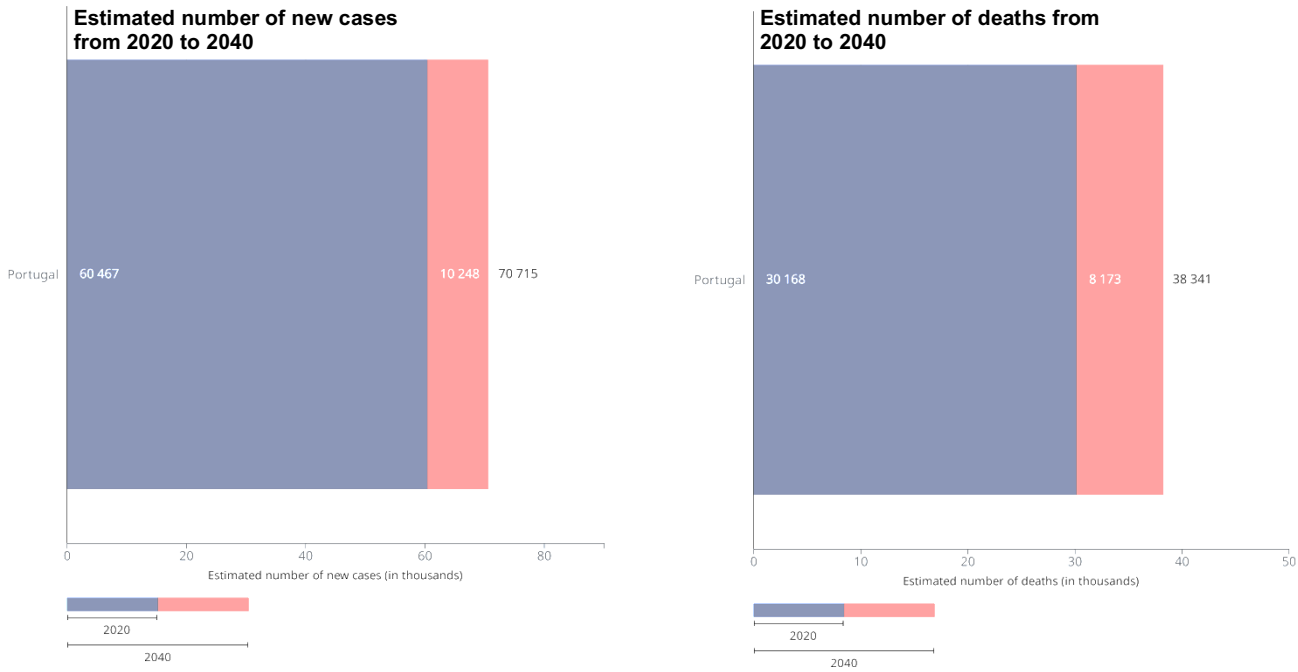


Figure 1: Estimated number of new cases and deaths from 2020 to 2040, both sexes, age 0-85 for all cancers. Adapted from Global Cancer Observatory: Cancer Tomorrow. 2020. (https://gco.iarc.fr/tomorrow/en/dataviz/bars?populations=620&single_unit=5000&mode=population&types=0)

2. HUMAN PAPILLOMAVIRUS AND CANCER

Papillomaviridae is one of the most ancient viral families, with at least 350 million years old [5,6]. Papillomaviruses (PVs) comprise a diverse group of viruses infecting several animal species, such as fishes, birds, mammals and reptiles [6,7]. Changes in the epithelium of their ancestral host (the first reptiles) seems to be linked to the origin of these viruses [6]. PVs are non-encapsulated viruses with 50-60nm in diameter presenting a small circular double-stranded deoxyribonucleic acid (DNA) genome of nearly 8000 bp [5,7,8]. More than 300 PVs have been identified and sequenced, including 200 capable of infecting humans [9].

Human Papillomaviruses (HPVs) are contained in five evolutionary branches with different epithelial tropism and disease associations, including the Alpha-, Beta-, Gamma-, Nu- and Mu- papillomaviruses (α, β, γ, μ and ν, respectively) [6,10]. Alpha-, Beta- and Gamma-papillomaviruses are the largest groups [9]. Beta- and Gamma-HPVs are capable to cause chronic unapparent infections, leading to the virion's production without evident damage to the host [10]. Alpha-papillomaviruses are mainly divided into two groups: the low-risk types (e.g. HPV-6 and -11) associated with warts and benign lesions development; and the high-risk types (e.g. HPV-16 and -18) associated with the development of some types of cancer [10] – **Figure 2**.

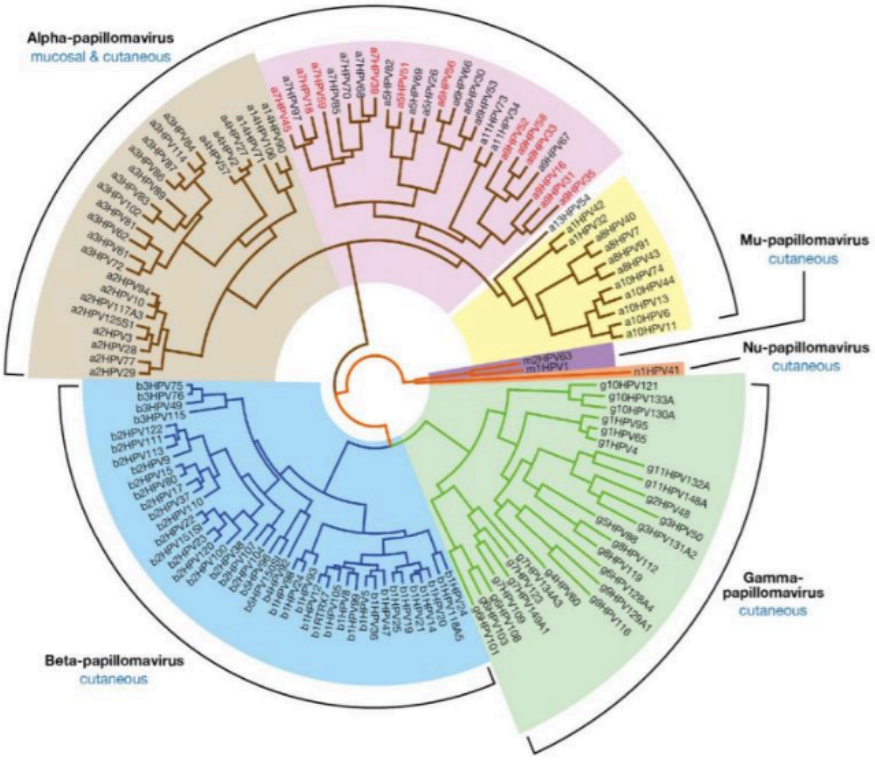


Figure 2: Evolutionary relationships between the five HPV genera. Alpha-papillomaviruses are classified as low-risk (cutaneous – brown – or mucosal – yellow) or as high-risk (pink) in accordance to their association

with cancer development. The high-risk types highlighted in red are confirmed as “human carcinogens” and the remaining high-risk types are “probable” or possible” carcinogens. Adapted from Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. Human papillomavirus molecular biology and disease association. *Rev Med Virol.* 2015;25 Suppl **1**:2-23.

In 1974, Harald zur Hausen identified for the first-time the HPV as the etiological factor of cervical cancer, and since 1995 multiple authors have been describing the different HPV genotypes in accordance to the risk of carcinogenesis [11-15] – **Figure 3**. The critical event for developing high-grade cervical lesions that can progress to Invasive Cervical Cancer (ICC) is the persistent High-risk-HPVs (Hr-HPVs) infections [14,16].

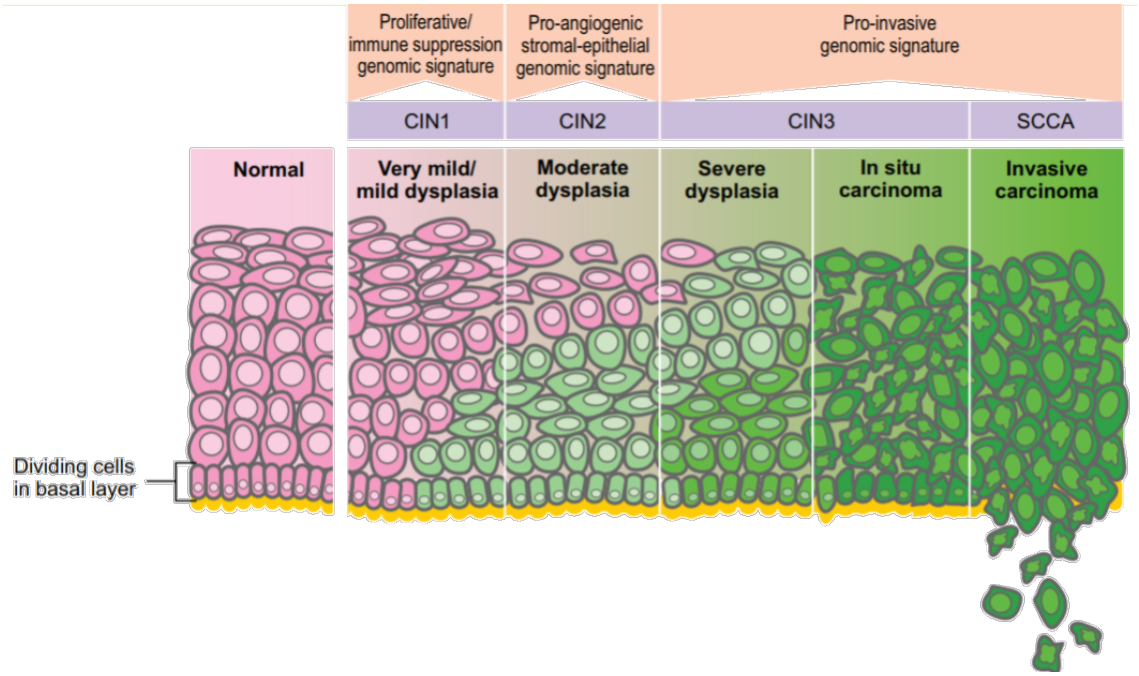


Figure 3: Cervical Cancer HPV-associated carcinogenesis mechanism. Adapted from https://home.ccr.cancer.gov/inthejournals/archives/gius

To this moment, more than 200 different HPV genotypes are recognized [11,14], however, the subtypes HPV-16 and HPV-18 cause the majority of cervical cancer cases worldwide and combined with HPV-31, -33, -45, -52 and -58 account for more than 90% of all cases [16-20]. There are fourteen Hr-HPV genotypes (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68) included in most marketed HPV tests and their identification has been useful for the development of HPV vaccines and in the implementation of cervical cancer prevention strategies [21-24].

HPV infection is commonly transmitted by sexual contact, afflicting the majority of sexually active population of both sexes [10,14]. The Hr-HPV infection is associated with virtually all cervical cancers and a significant proportion of other anogenital (vagina, vulva, anus and penis) and head and neck cancers, namely

in the oropharynx (tonsils and base of the tongue), oral cavity and larynx [14, 25-29]. Controversial associations between Hr-HPV infection and other cancers, such as lung, oesophageal and breast cancers, have been described [30-37].

3. CERVICAL CANCER

Worldwide in 2020, Cervical Cancer was the fourth most frequently diagnosed cancer and the fourth main cause of cancer death in women. It has been responsible for 604,127 new cases and 341,381 deaths, in accordance with Globocan [38]. In Portugal, in 2020 it was estimated a total of 865 new cases and 379 deaths, with an Age-Standardized incidence rate of 10.7 and an Age-Standardized mortality rates of 3.7 per 100,000 women [38] – **Figure 4**.

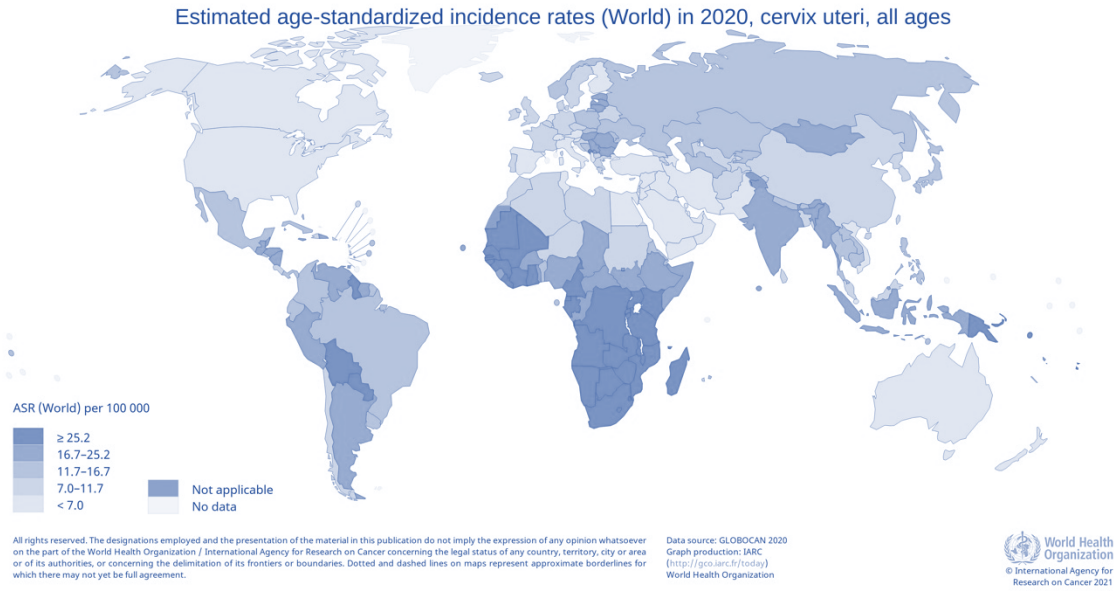


Figure 4: Epidemiology of Cervical Cancer in the world. Adapted from Sung, H., et al., Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians, 2021. 71(3): p. 20

The guidelines for cervical cancer screening are based on the principle of prevention, as it is divided into two stages: primary prevention strategies with the implementation of HPV vaccines (2-valent with recombinant proteins L1 of HPV with -16 and -18 genotypes; 4-valent with recombinant proteins L1 of HPV with -6, -11, -16, -18 genotypes; or 9-valent with recombinant proteins L1 of HPV with -6, -11, -16, -18, -31, -33, -45, -52 and -58 genotypes), and secondary prevention by cervical cancer screening measures [16,39-41].

In Portugal, Cervical Cancer Screening was implemented in 1978 and in 1990 it began to be partially organized in the Central Region of the country [42]. Cervical Cancer Screening programs are currently implemented in all health regions, with a geographic coverage of 88% of all Primary Care Units in Mainland Portugal and in the Azores, for asymptomatic women aged ≥ 25 and ≤ 60 years [43]. In Mainland Portugal, the Regional Health Administrations (ARS) are responsible for identifying the eligible population, which is subsequently invited to participate by the family doctors responsible for their follow-up, and the Director of the Clinical Council of the Health Centres Grouping (ACES) is responsible for inviting women who do not have a family doctor to be screened [43].

In 2009, the North Region of Portugal started its Regional Cervical Cancer Screening (RCCU) which was progressively expanded to the entire region [43]. The strategy for RCCU was liquid-based cytology and HPV-test as reflex-test in case of Atypical Squamous Cells of undetermined significance (ASC-US) [43]. In 2016, a pilot study using full Hr-HPV genotyping as the primary approach for cervical cancer screening was implemented in the Northern Region of Portugal [44].

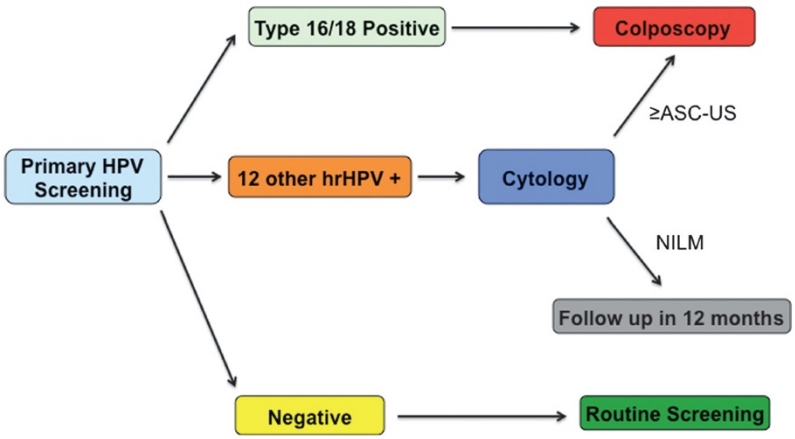


Figure 5: Flowchart of Cervical Cancer Screening Program in Portugal. Negative HPV test repeats screening in five years; Positive cases for HPV-16 and -18 are referred to colposcopy evaluation; Positive cases for any other Hr-HPV genotype undergo reflex cytology and if positive for higher than ASC-US are referred to colposcopy evaluation or if negative for intraepithelial lesion malignancy (NILM) repeats HPV test in one year. Adapted from Huh, W.K., et al., Use of primary high-risk human papillomavirus testing for cervical cancer screening: interim clinical guidance. Obstet Gynecol, 2015. 125(2): p. 330-337.

With an average eligible population in the Northern Region of Portugal of 193,493 women/year, the population coverage rate was 65.0% in 2019 and 26.9% in 2020. The rate of adherence was 87.3% in 2019 and 85.5% in 2020, with a total of 109,725 women screened in 2019 and 44,194 in 2020 [43]. This screening enables the detection and treatment of pre-malignant lesions and the early detection and treatment of cervical cancer with a consequent impact on reducing incidence and mortality [43].

4. VAGINAL MICROBIOME AND HPV

One of the eminent research topics in health is the utility of microbiome analysis in disease development and prevention, including the role of the microbiome in HPV-induced diseases [45]. The generation of reproducible microbiome archetypes, or community state types (CSTs) [46] most commonly found in patients with cervical lesions were CSTs characterized by *Lactobacillus* depletion, predominance of anaerobic bacteria and dominance of *Lactobacillus iners* [47-50]. *Sneathia* was significantly enriched in cervical lesions samples in multiple studies, and its presence was associated with alterations in immune mediators [49,51]. A study of the microbiome in cervical-vaginal samples from the Portuguese population, by Jani Silva *et al*, suggests that genital *Ureaplasma urealyticum*, *U. parvum* and *Mycoplasma hominis* are common in Portuguese women of reproductive age (<30 years old) while *M. genitalium* is rare in the same population [52]. Thus, we can infer the importance of studying the vaginal microbiome and its possible influence on HPV infections and its outcome.

AIMS

With this study we intend to describe the patterns of multiple Hr-HPVs infections and microbiome in the population and evaluate its impact on cervical disease development. The data provided by the full Hr-HPV genotyping is of great interest not only from the epidemiological point of view, but also to understand better how the different Hr-HPVs may correlate in the development of cervical lesions/cancer. This is highly important, especially when considering the high frequency of multiple infections, and to better understand what is the role of the different Hr-HPVs in the context of multiple infections in the development of cervical lesions/cancer. Hence, there is an increasing interest in analyse the data from full Hr-HPV genotyping to be able to develop better and more accurate strategies for cervical cancer screening.

The Specific aims are:

1. Description of multiple Hr-HPV infections in cervical cancer screening samples;
2. Assess the impact of different Hr-HPV genotypes on multiple infections;
3. Characterize the microbiome of Hr-HPV positive cervical samples;
4. Correlate the information between multiple Hr-HPV infections, microbiome and cytological diagnosis.

CHAPTER II

IMPACT OF HIGH-RISK HUMAN PAPILLOMAVIRUS GENOTYPING IN CERVICAL DISEASE IN THE NORTHERN REGION OF PORTUGAL: REAL WORLD DATA FROM REGIONAL CERVICAL CANCER SCREENING PROGRAM

AUTHORS

Andreia Rosário [1,2], Ana Sousa[1], Joana Marinho-Dias[2], Rui Medeiros[1,2,4], Cláudia Lobo[3], Luís Leça[3], Fernando Tavares[6], Gabriela Martins [2], Paula Monteiro [3,7], Rui Henrique[3,5,7], Hugo Sousa[1,2]

AFFILIATIONS

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

[2] Clinical Pathology Service, Clinical and Medical Pathology Department, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center (Porto.CCC), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

[3] Pathological Anatomy Service, Clinical and Medical Pathology Department, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center (Porto.CCC), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

[4] Research Department, Portuguese League Against Cancer (LPCC-NRNorte), Estrada Interior da Circunvalação 6657, 4200-172 Porto, Portugal

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

[6] Administração Regional de Saúde do Norte I.P., Rua de Santa Catarina 1288, 4000-477 Porto, Portugal

[7] Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar – University of Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

CORRESPONDENCE TO

Hugo Sousa, MD, PhD

Clinical Pathology Service, Clinical and Medical Pathology Department

Portuguese Oncology Institute of Porto FG EPE

Rua Dr. António Bernardino Almeida

4200-072 Porto, PORTUGAL

Phone: +351 22 508 4000 (ext 5410)

Fax: +351 22 508 4001

E-mail: hugo.sousa@ipoporto.min-saude.pt

CONFLICT OF INTEREST

All authors declare no conflict of interest.

ABSTRACT

Cervical Cancer prevention strategy is based on the vaccination and screening. In Portugal, Cervical Cancer Screening is performed by HPV-test with reflex cytology since 2017. Here we characterize the prevalence and distribution of Hr-HPV genotypes and cytological abnormalities in cervical samples collected in the Regional Cervical Cancer Screening Program from the Northern Region of Portugal between August 2016 and December 2021.

A total of 462.401 women with mean age 43.73 ±10.79 years old (median age 45; range 24-66) were enrolled in the analysis. Overall, we observed that 12.5% of all cases were Hr-HPV positive, with a prevalencevarying according to age from a maximum of 20.8% at age 25 to a minimum of 8.3% at age 64. The 5 most common Hr-HPV genotypes found in our population were HPV-68 (16.09%), HPV-31 (15.3%), HPV-52 (13.33%), HPV-51 (12.96%) and HPV-16 (11.06%). Age-related Hr-HPV genotype distribution shows that HPV-31 predominated in women of 30-40 years whereas HPV-68 predominated in women of 45-64 years. Cytological analysis of Hr-HPV positive cases revealed 65.68% NILM, 20.83% ASC-US, 8.86% LSIL, 1.65% HSIL, 2.85% ASC-H, 0.09% AGC, 0.01% AdC, and 0.01% ICC. The analysis also showed that Hr-HPVs included in the 9-valent vaccine (HPV-9val) represented 52.13% of NILM, 59.21% of ASC-US, 55.06% of LSIL, 90.14% of HSIL, 83.50% of ASC-H, 100% of ICC, 83.02% of AGC and 77.78% of AdC.

This is the largest study on Hr-HPV genotyping for Cervical Cancer Screening which included data from a period of over 5 years (65 months) and its interpretation adds important information for Cervical Cancer prevention strategy. Overall, we confirm that Hr-HPV genotypes present in the 9-valent vaccine are responsible for the large majority of cervical lesions and all cancers, which reinforces the utility of this vaccine in the strategy for cervical cancer elimination in our population.

KEYWORDS: Human Papillomavirus, cervical cancer, screening, genotyping, prevalence, epidemiology.

1. INTRODUCTION

Worldwide, Invasive Cervical Cancer (ICC) is one of the most important cancers in women, being responsible for 569,847 new cases and 311,365 related deaths in 2018, according to Globocan [1]. In Portugal, in 2018 it was estimated a total of 750 new cases and 350 deaths, with an Age-Standardized incidence and mortality rates of 8.9 and 2.8 per 100,000 women, respectively [1].

Since 1970s that Human Papillomavirus (HPV) were identified as the etiological factor of cervical cancer, and since 2005 that The International Agency for Research on Cancer (IARC)consider 14 different HPVs as carcinogens, which are described now as High-Risk HPVs (Hr-HPVs) [2–5]. It is well known that the persistent infection by Hr-HPVs is associated with the development of high-grade cervical lesions that can evolve to ICC [5,6]. There are over 150 different HPV genotypes [3], nevertheless HPV-16 and HPV-18 are responsible for the majority of cervical cancer cases worldwide, and together with HPV-31, -33, -45, -52 and -58 represent over 90% of all cases [6–10]. There are 14 Hr-HPV genotypes (HPV 16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68) included in the majority of HPV-tests commercialized and its identification may be useful for the development of future HPV vaccines or cervical cancer prevention strategies [11–13].

Cervical cancer is actually a preventable cancer since there are both primary prevention measures with the implementation of vaccines against HPV, and with secondary prevention by cervical cancer screening strategies [6,14–16]. In Portugal, cervical cancer screening was introduced back in 1978 as an opportunistic strategy and in 1990 it started to be partially-organized in the Centre region of the country [17]. Due to the division of the country in different regional health administrations, the cervical cancer screening program was progressively implemented in the Alentejo, Algarve, Azores and the Northern region of Portugal. In the Northern region of Portugal cervical cancer screening started in 2009 and progressively extended to the whole region, using liquid-based cytology and HPV-testing as a reflex in cases of Atypical Squamous Cells of Undetermined Significance (ASC-US). In 2016, a pilot study was implemented in the North Region of Portugal using Hr-HPV full genotyping as the primary method for cervical cancer screening. In this study we report the results of that initiative which encompassed Hr-HPV full genotyping in women attending the Regional Cervical Cancer

Screening Program (RCCU) in the Northern region of Portugal and discuss its potential impact in the definition of and monitoring of the HPV vaccination program.

2. MATERIAL AND METHODS

2.1 STUDY POPULATION

The RCCU is an organized screening program designed to meet all women aged 25–60 years old (with extension up to 64 years of age) from the North Region of Portugal. The current study reported the data at 5-year intervals regarding Hr-HPV genotyping and liquid-based cytology (LBC) samples from all cases processed and analysed between August 2016 and December 2021. This study was approved by the Institutional Ethical Committee (ref. CES-IPO:146/022).

2.2 HPV GENOTYPING

Hr-HPV genotyping was performed according to the manufacturer's instructions in liquid-based cytology samples with *Anyplex™ II HPV HR Detection* (Seegene®, Seoul, Korea). This kit is composed by an automated system for deoxyribonucleic acid (DNA) isolation (Nimbus IVD or STARlet from Hamilton), a Real-Time Polymerase Chain Reaction (PCR) system (CFX96 PCR from Bio-Rad) and data analysis software (Seegene Viewer™ from Seegene®). This method, already validated for use in cervical cancer screening allows for simultaneous detection and genotyping of 14 Hr-HPV subtypes, including HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68 plus an internal control (human beta-globin) in a single reaction. All reactions include positive and negative controls provided in the kit.

2.3 CYTOLOGICAL EVALUATION

Hr-HPV positive cases undergo cytopathological examination of all cases, performed by dedicated cytopathologists using cytology information according to the Bethesda Classification (Modified) using the following terminology [18]: Negative for Intraepithelial Lesion or Malignancy (NILM); ASC-US; Atypical Squamous Cells that cannot exclude an HSIL (ASC-H); Low-Grade Squamous Intraepithelial Lesions (LSIL); High-Grade Squamous Intraepithelial Lesions (HSIL);

ICC; Atypical Glandular Cells (AGC) and Adenocarcinomas (AdC). Thin Prep® Pap Test™ (Cytyc Corp., Boxborough, MA) for the detection of squamous epithelial characteristics and abnormalities.

2.4 STATISTICAL ANALYSIS

Statistical analysis was performed by descriptive statistics (frequencies/prevalence) and the correlation was performed using the computer software IBM SPSS Statistics for Mac, Version 24.0 (Armonk, NY: IBM Corp). The overall Hr-HPVs prevalence data was described by frequencies, percentages and age (continuous variable), using Student's t-test and ANOVA considering a statistical significance of 5% ($p<0.05$). We described the patterns of Hr-HPVs infection in the population and correlated the data with the cytological information.

3. RESULTS

A total of 462,401 women between 1 August 2016 and 31 December 2021 (mean age 43.73 ±10.79 years old; median age 45; range 24-66) were enrolled. Age and geographical distribution of women is depicted in **Table 1**.

Table 1 – Characterization of cervical cancer screening population (age groups, period of time and geographical location).

		Age Group								
	Total (n)	25 (n)	30 (n)	35 (n)	40 (n)	45 (n)	50 (n)	55 (n)	60 (n)	64 (n)
	462401	40501	47276	54110	64519	69447	67088	62805	51428	5227
Period										
2016	16442	1145	1496	1970	2382	2574	2659	2190	1823	203
2017	88280	7034	8856	10671	13138	13574	13011	11580	9255	1161
2018	101010	8044	10413	12224	14290	15096	14848	13654	11219	1222
2019	94666	8726	9687	11121	13205	14295	13445	12846	10543	798
2020	55827	5714	6201	6548	7518	8087	7497	7313	6413	536
2021	106176	9838	10623	11576	13986	15821	15628	15222	12175	1307
Geographical Location										
Aveiro	43093	3860	4008	4872	5650	6541	6468	6231	5099	364
Braga	135013	12286	13973	16093	19606	20830	19416	17535	13957	1317
Bragança	11858	880	1161	1327	1583	1604	1656	1756	1808	83
Porto	202507	17504	21368	23586	27985	30387	29617	27332	21777	2951
Viana do Castelo	33278	2975	3309	4153	4793	4965	4638	4375	3830	240
Vila Real	22975	1821	2159	2548	3158	3213	3289	3429	3202	156
Viseu	13629	1170	1291	1527	1740	1899	1996	2140	1750	116
missing data	*48*	*5*	*7*	*4*	*4*	*8*	*8*	*7*	*5*	*0*

3.1 HPV prevalence and genotyping

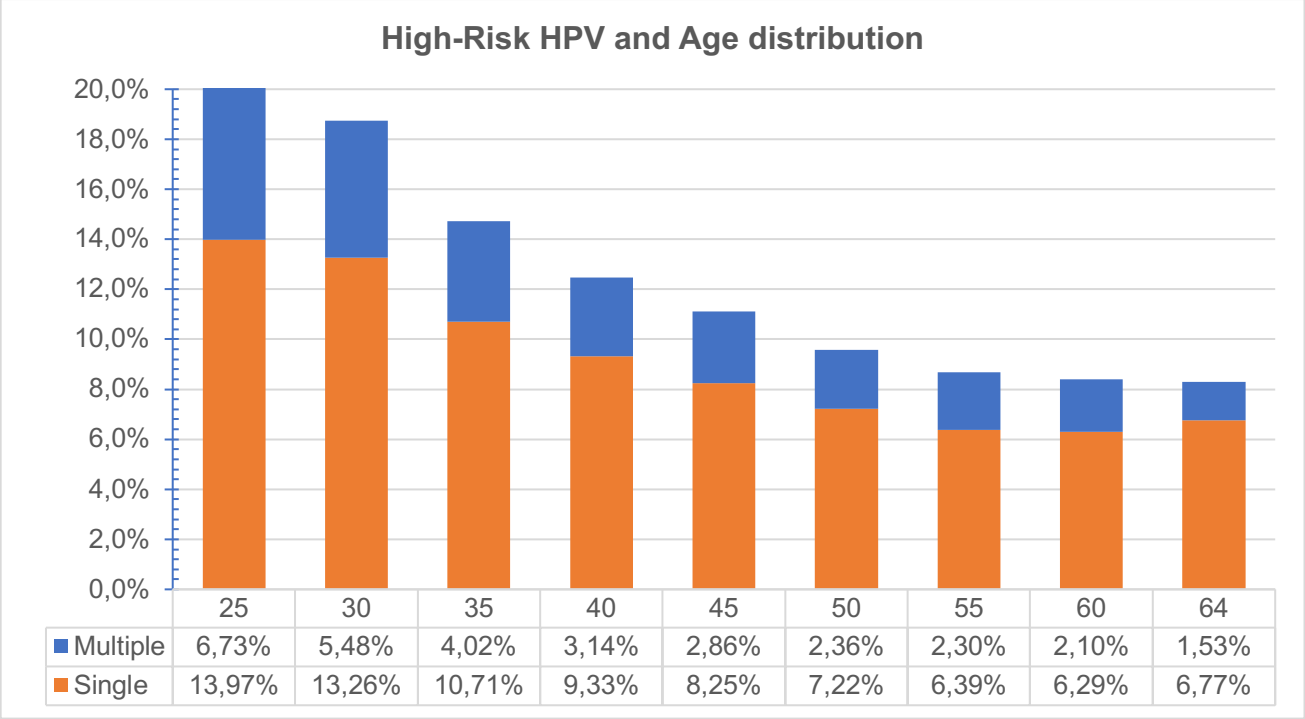
From the 462,401 cases included in the study, 57,796 (12.5%) were Hr-HPV positive, 32 cases were inconclusive (0.01%) and 358 (0.1%) had insufficient sampling for analysis – **Table 2**. Hr-HPV infection varied according to age ranging from a maximum of 20.8% at age 25 to a minimum of 8.3% at age 64; while regarding geographical location of women, Hr-HPV did not differ significantly, ranging from 10.6% to 13.2% – **Figure 1** and **Table 2**. Multiple infections (by two or more Hr-HPVs) were detected in 15,687 (27.14%) women, ranging from 18.39% at age of 64

to 32.43% at age of 25; without a significant variation regarding geographical location (range 24.76% to 28.03%) – **Figure 2** and **Table 2**. HPV-16 and -18 were detected in 8,321 (14.4%) women, ranging from 3.92% at age of 25 to 7.58% at age of 35; without a significant variation regarding geographical location (range 12.2% to 15.09%) – **Figure 3** and **Table 2**.

The Hr-HPVs that were included in the 9-valent vaccine (HPV-9val) were detected in 32,002 (55.37%) women, ranging from 47.94% to 59.46% within the different age groups and no significant variation was observed regarding geographical location (range 53.96% to 55.92%) – **Figure 4** and **Table 2**.

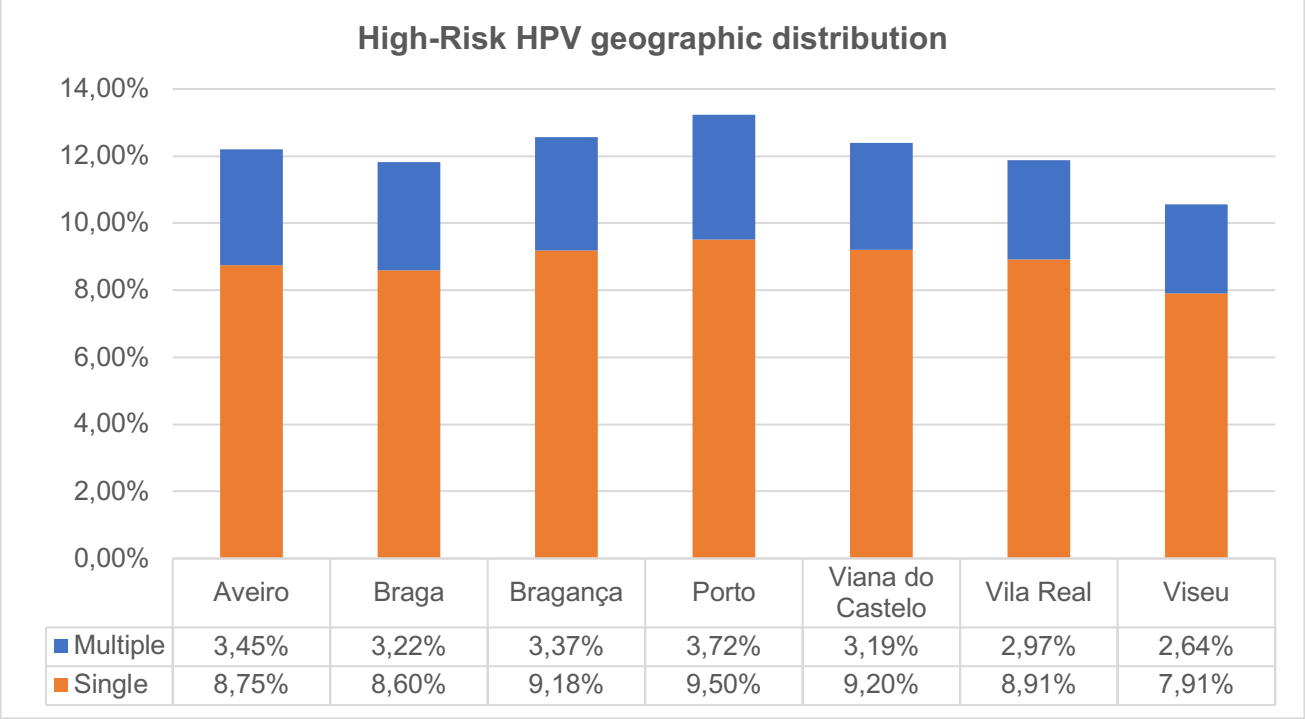
Table 2 – Hr-HPV prevalence according to age groups and geographical location.

	Total (n)	HPV Positive n (%)	HPV Multiple Infection n (%)	HPV-16/-18 n (%)	HPV-9val n (%)
	462401	57796	15687	8321	32002
Age Group					
25	40501	8408	2727	330	4031
30	47276	8889	2591	1385	5112
35	54110	8009	2175	1408	4762
40	64519	8070	2029	1324	4542
45	69447	7745	1983	1350	4389
50	67088	6444	1581	962	3503
55	62805	5472	1442	838	3027
60	51428	4324	1079	674	2410
64	5227	435	80	50	226
Geographical Location					
Aveiro	43093	5258	1474	764	2935
Braga	135013	15954	4289	2320	8868
Bragança	11858	1489	397	197	825
Porto	202507	26786	7435	3928	14832
Viana do Castelo	33278	4126	1054	562	2274
Vila Real	22975	2729	677	333	1480
Viseu	13629	1438	356	217	776



	25	30	35	40	45	50	55	60	64
Multiple	6,73%	5,48%	4,02%	3,14%	2,86%	2,36%	2,30%	2,10%	1,53%
Single	13,97%	13,26%	10,71%	9,33%	8,25%	7,22%	6,39%	6,29%	6,77%

Figure 6 - High-risk HPV and age distribution.



	Aveiro	Braga	Bragança	Porto	Viana do Castelo	Vila Real	Viseu
Multiple	3,45%	3,22%	3,37%	3,72%	3,19%	2,97%	2,64%
Single	8,75%	8,60%	9,18%	9,50%	9,20%	8,91%	7,91%

Figure 7 - High-risk HPV geographic distribution.

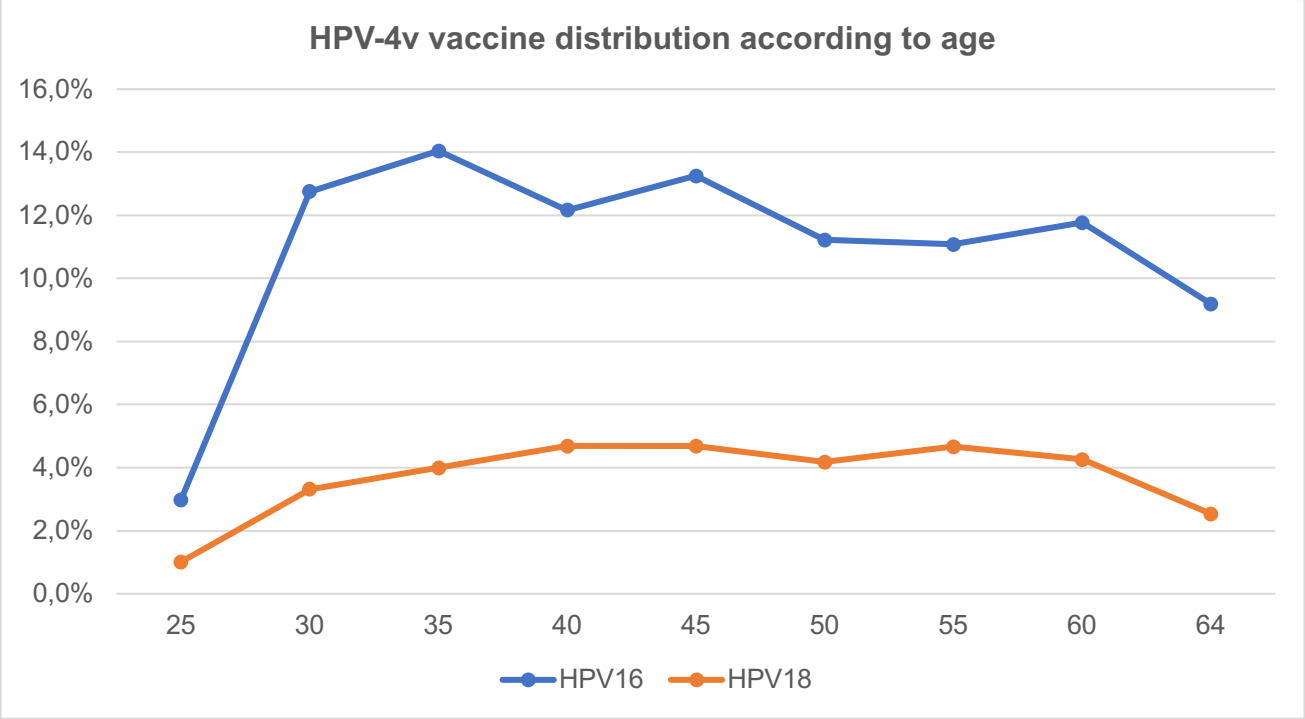


Figure 8 - HPV-16 and -18 distribution according to age.

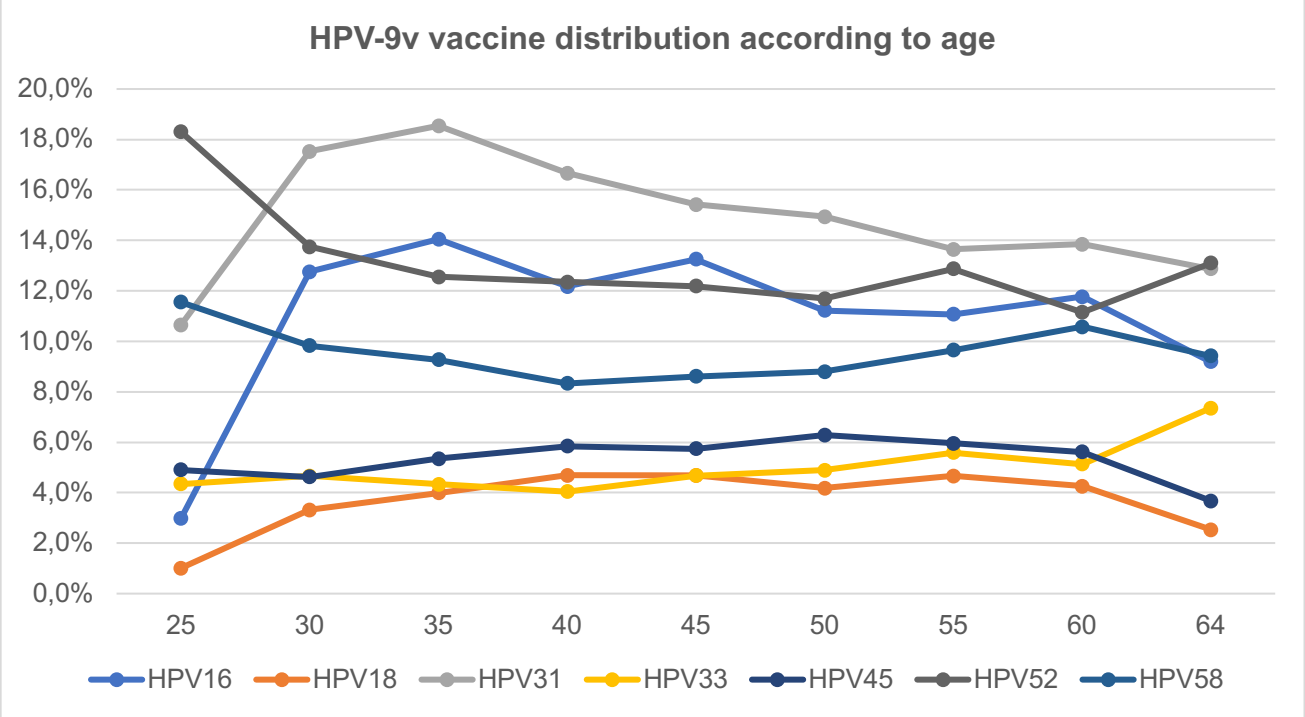


Figure 9 - HPV-9valent vaccine distribution according to age.

The 5 most common Hr-HPV genotypes found in our population were HPV-68 (16.09%), HPV-31 (15.3%), HPV-52 (13.33%), HPV-51 (12.96%) and HPV-16 (11.06%) – **Figure 5** and **Table 3**. Hr-HPV distribution according to age revealed that HPV-31 predominated in women 30-40 years whereas HPV-68 predominated in women 45-64 years – **Figure 6** and **Table 3**. The most common Hr-HPV genotypes had similar patterns in all geographic locations – **Figure 7** and **Table 3**.

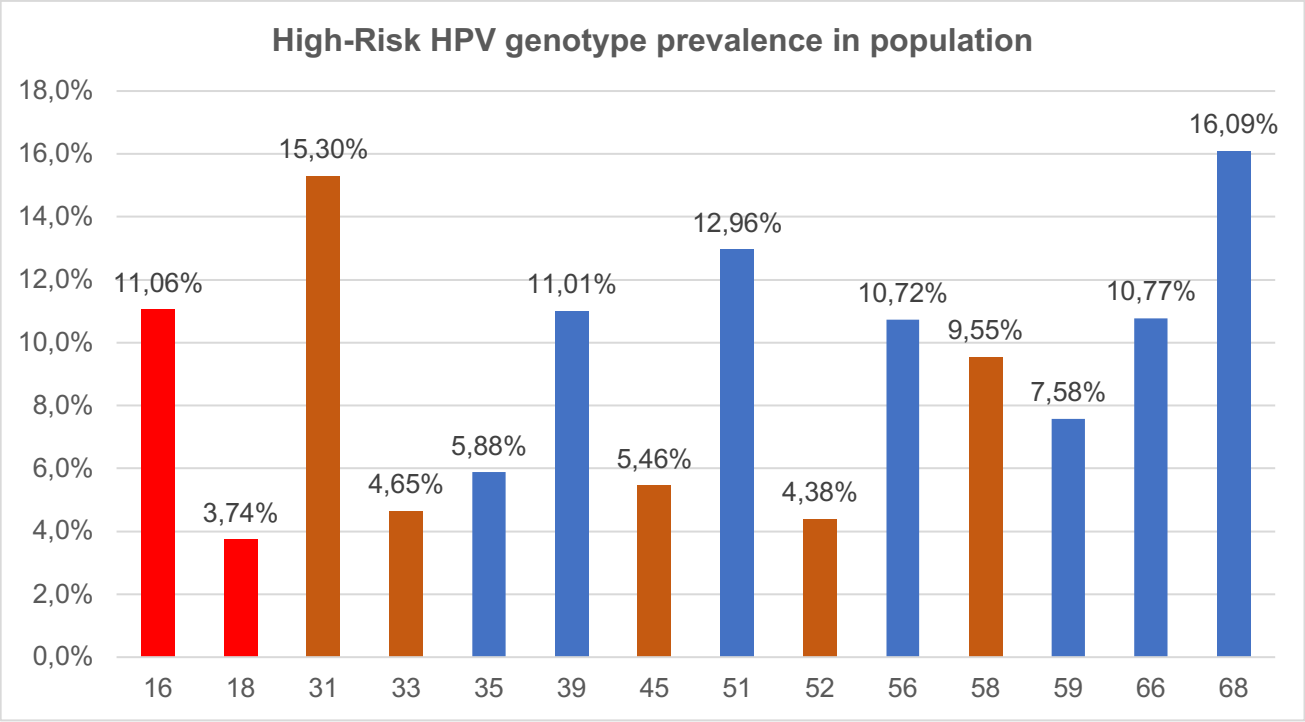


Figure 10 - High-Risk HPV genotype prevalence in population.

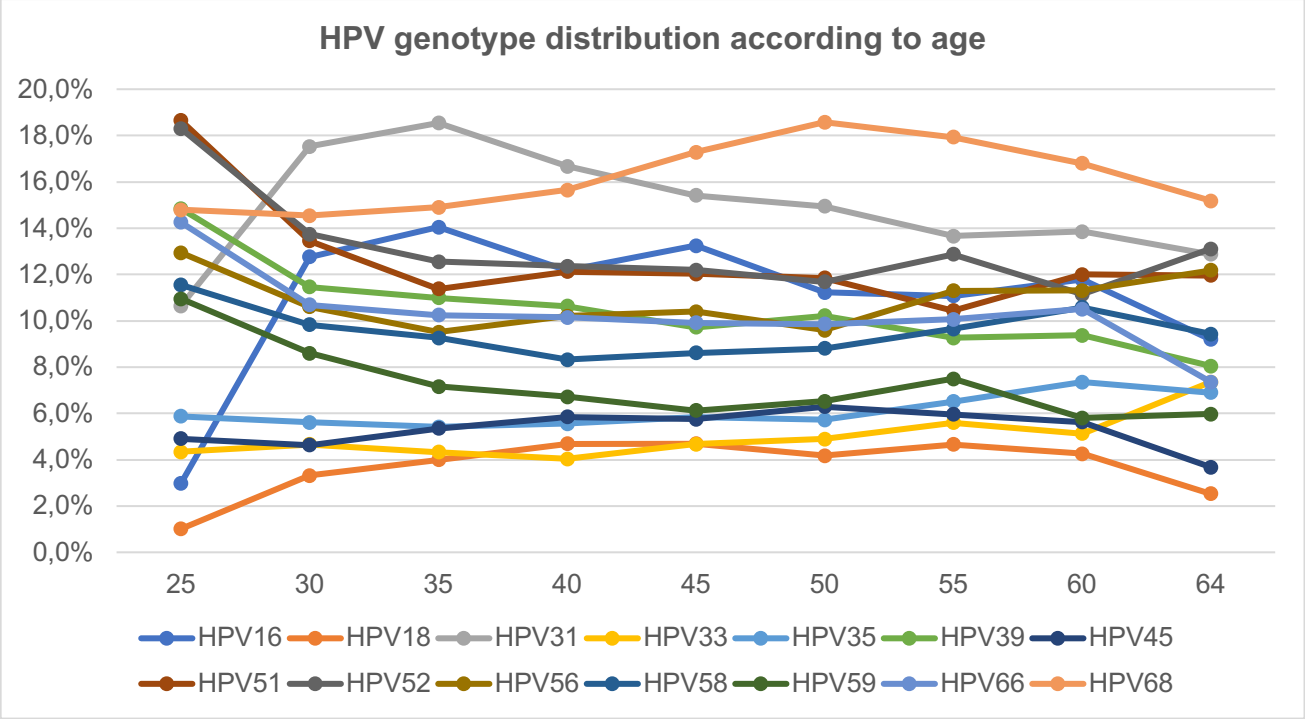


Figure 11 - HPV genotype distribution according to age.

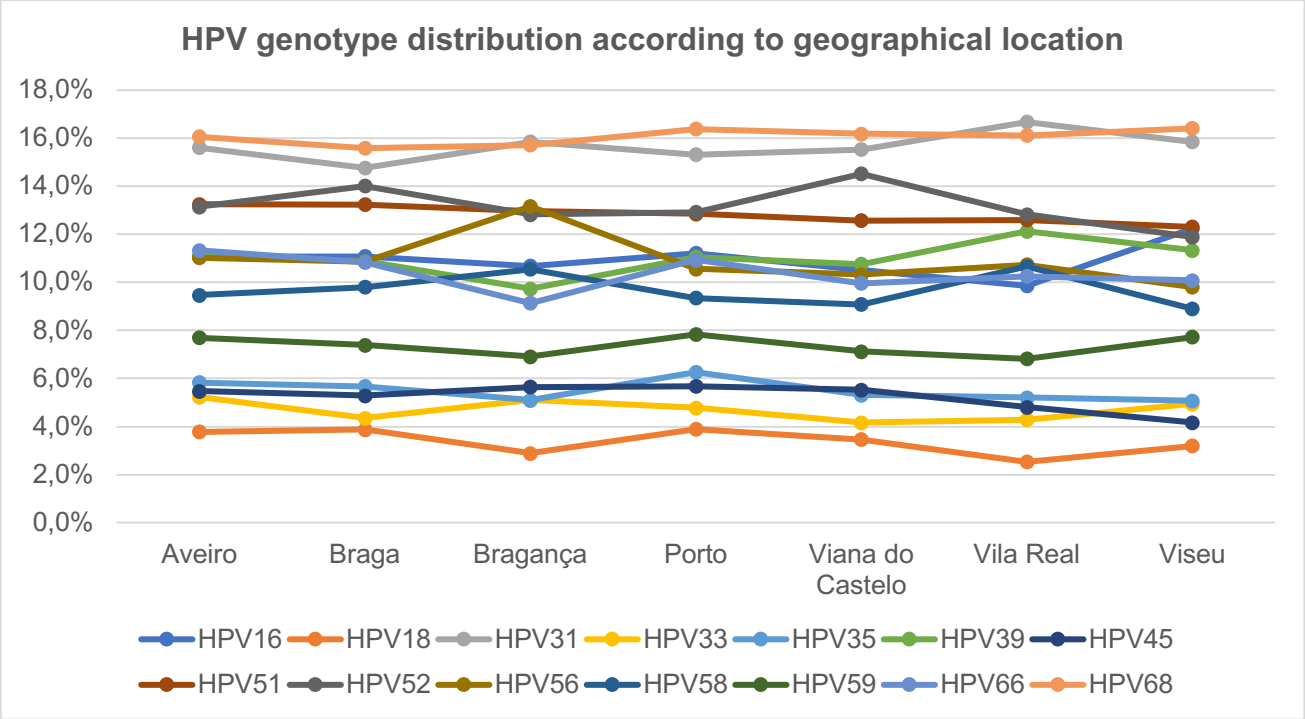


Figure 12 - HPV genotype distribution according to geographical location.

Table 3 – Cytological distribution and correlation with age and Hr-HPV genotypes.

		NILM	ASC-US	LSIL	HSIL	ASC-H	ICC	AGC	Adc
Age Group									
TOTAL	57559	37854	11983	5074	943	1633	10	53	9
25	8415	5583	1698	948	59	126	0	1	0
30	8891	5614	1909	893	164	304	0	7	0
35	7959	4997	1730	703	191	322	0	10	6
40	8044	5101	1776	729	173	252	2	10	1
45	7712	4946	1668	718	131	236	1	11	1
50	6415	4217	1335	555	119	184	2	3	0
55	5425	3870	1029	343	59	115	2	6	1
60	4261	3189	762	181	43	81	2	3	0
64	435	337	76	4	4	13	1	0	0
Hr-HPV genotypes									
Total	57100	37506	11893	5056	943	1630	10	53	9
Multiple Infections	15549	8416	3968	2251	331	568	0	12	3
HPV-16 and -18	8201	4274	1984	795	490	620	9	23	6
HPV 9-val genotypes	31650	19552	7042	2784	850	1361	10	44	7
-16	6307	3143	1526	617	453	545	9	11	3
-18	2126	1230	525	202	56	98	0	12	3
-31	8745	5293	2037	818	178	402	1	14	2
-33	2667	1478	651	288	102	145	0	2	1
-35	3353	2010	776	370	66	130	0	1	0
-39	2822	1672	762	320	31	35		2	0
-45	3115	2061	664	263	39	85	0	2	1
-51	7423	4370	1733	1053	68	193	0	5	1
-52	7639	4930	1658	680	120	244	0	6	1
-56	6129	3718	1286	983	45	96	0	0	1
-58	5463	3307	1301	576	104	172	0	3	0
-59	4337	2778	993	454	37	70	0	4	1
-66	6159	3820	1301	910	35	90	0	3	0
-68	9217	6469	1856	697	59	134	0	2	0

3.2 Cytological distribution and HPV genotyping

Of all Hr-HPV positive cases, cervical cytology was available for 57.103 cases of which 37.506 (65.68%) were NILM. Of the cases with abnormal cytologies we found 11.895 (20.83%) women with ASC-US, 5.057 (8.86%) women with LSIL, 943 (1.65%) women with HSIL, 1630 (2.85%) women with ASC-H; 53 (0.09%) women with AGC, 9 (0.01%) women with AdC, and 10 (0.01%) women with ICC **-** Table 3**.**

According to the age, the minimum of NILM cases is 62.78% at 35 years old and the maximum is 77.47% at 64 years old, for ASC-US is 17.47% at 64 years old and 22.08% at 40 years old, for LSIL is 0.92% at 64 years old and 11.27% at 25 years old, for HSIL is 0.70% at 25 years old and 2.40% at 35 years old, for ASC-H is 1.50% at 25 years old and 4.05% at 35 years old, for AGC is 0.00% at 64 years old and 0.14% at 45 years old, for AdC is 0.00% at 25, 30, 50, 60 and 64 years old and 0.08% at 35 years old and for ICC is 0.00% for 25 to 35 years old and 0.23% at 64 years old – **Figure 8** and **Table 3**.

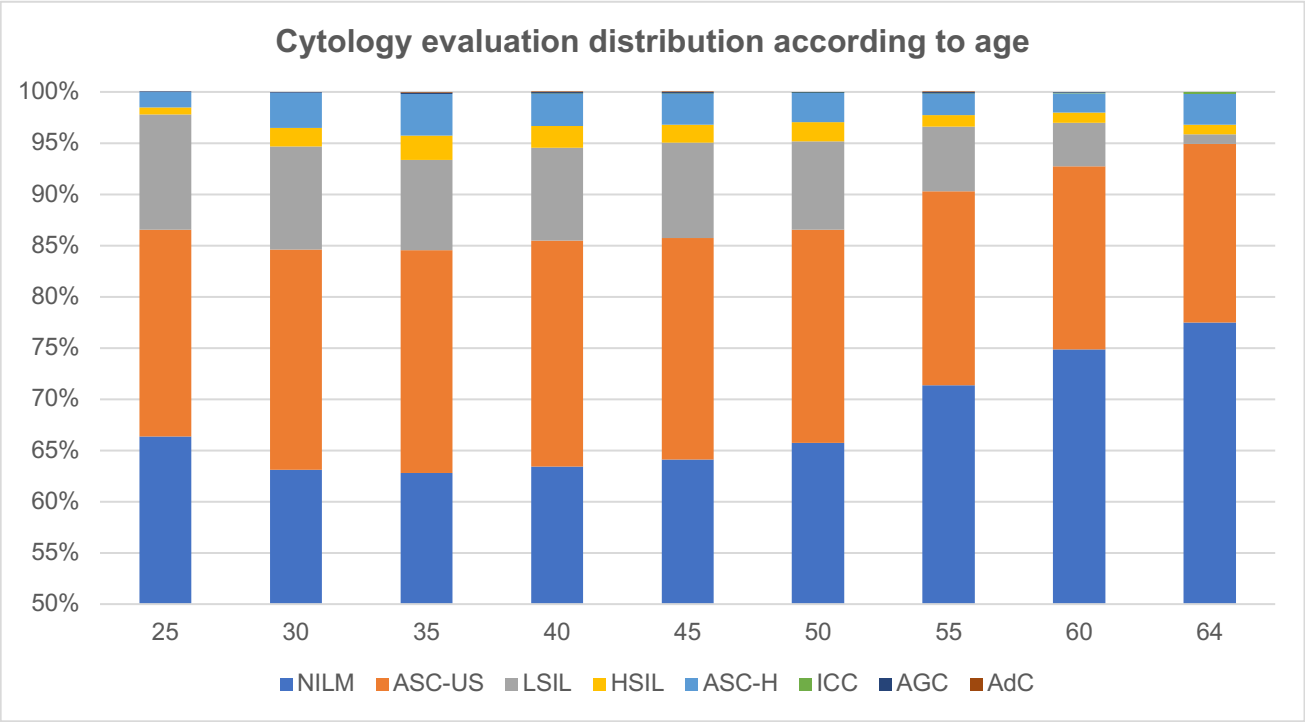


Figure 13 - Cytology evaluation distribution according to age.

Regarding the correlation between the different Hr-HPV genotypes and cytological distribution, we observed that Multiple Hr-HPV infections represented 22.44% of NILM, 33.36% of ASC-US, 44.52% of LSIL, 35.10% of HSIL, 34.85% of ASC-H, 0% of ICC, 22.64% of AGC and 33.33% of AdC – **Figure 9** and **Table 3**. HPV-16/-18 infections represented 11.40% of NILM, 16.68% of ASC-US, 15.72% of LSIL, 51.96% of HSIL, 38.04% of ASC-H, 90.00% of ICC, 43.40% of AGC and 66.67% of AdC - **Figure 9** and **Table 3**. Hr-HPVs included in the 9-valent vaccine (HPV-

9val) represented 52.13% of NILM, 59.21% of ASC-US, 55.06% of LSIL, 90.14% of HSIL, 83.50% of ASC-H, 100% of ICC, 83.02% of AGC and 77.78% of AdC - **Figure 9** and **Table 3**.

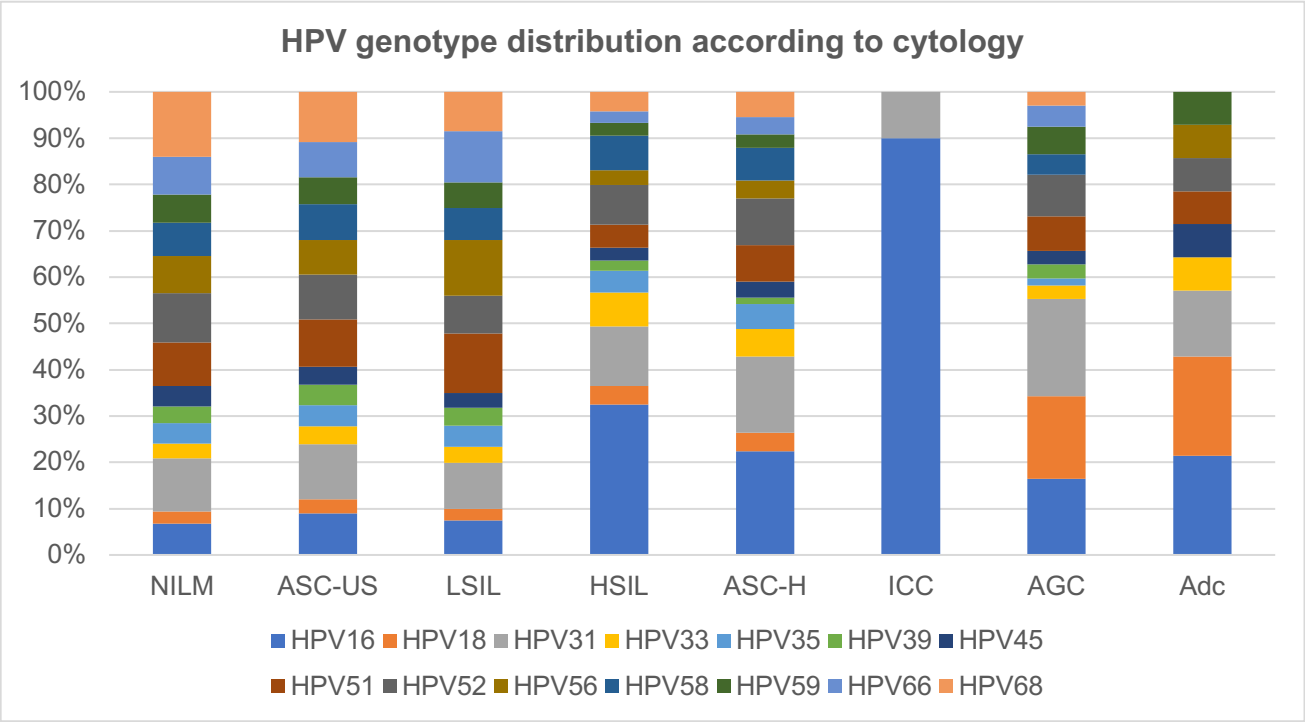


Figure 14 - HPV genotype distribution according to cytology.

4. DISCUSSION

The new strategies for cervical cancer screening support the use of HPV test as primary test (higher sensitivity) followed by triage of HPV-positive results with cytology (higher specificity) [19–22]. Literature describes that HPV DNA testing is more sensitive for identifying women with CIN 2+ compared with cytology, despite having a lower specificity [16,23–25]. Indeed, studies shown that in women aged 30 to 69 years the sensitivity of HPV test is around 95% compared with 55% for cytology [20].

Portugal has a high-incidence of cervical cancer when compared with the majority of European countries and therefore Portuguese governments have started with vaccination and more effective screening strategies since 2008. Since September 2017, the Portuguese Ministry of Health has decided that cervical cancer screening should be performed using HPV test as the primary screening method. Hr-HPV full genotyping has been used more often since it is considered to provide additional epidemiological data [26–28]. In this study we report the prevalence of Hr-HPV genotypes detected in samples from women attending the cervical cancer screening from the Northern region of Portugal over a period of 5 ears and combine the information regarding the cytological classification of the Hr-HPV positive cases [25,28,29].

Our results report an overall prevalence of Hr-HPV of 12.5% which is significantly different from the reported value (19.4%) in the CLEOPATRE study from Pista *et al.* [30] and the reported (17.8%) in the Catalan Institute of Oncology (ICO)/IARC Information Centre on HPV and Cancer [31]. Nevertheless, this prevalence is similar to the expected prevalence in the majority of European countries [31]. The data we have first reported between August 2016 and December 2017 discloses a prevalence of 10.2% ranging from a maximum of 17.1% at age 25 to a minimum of 6.2% at age 64, and with simultaneous infections by two or more Hr-HPVs to represent 25.7% (31.0% at age of 25 to 16.5% at age of 64) [32]. Currently, by analysing 5 years of data in RCCU, we observe different results: the Hr-HPV prevalence is 12.5% varying according to age from a maximum of 20.8% at age 25 to a minimum of 8.3% at age 64. Despite the changes, data corroborate published evidence that HPV infections are extremely more frequent in young women and tend to become much lower after the age of 45 [33]. Furthermore, actually the Hr-

HPV multiple infections rate is 27.14% and it is important to analyse the potential impact of this data since it is unclear what is the biological behaviour of multiple infections and its outcome.

Regarding the HPV genotypes, in our previous study we found that the most prevalent were HPV-16 (17.5%), HPV-39 (16.7%), HPV-31 (15.0%), HPV-68 (13.2%), HPV-52 (10.7%) and HPV-51 (10.6%) [32] and now the 5 most common Hr-HPV genotypes found in our population were HPV-68 (16.09%), HPV-31 (15.3%), HPV-52 (13.33%), HPV-51 (12.96%) and HPV-16 (11.06%). Worldwide, the five most prevalent types are HPV-16, followed by HPV-18, HPV-52, HPV-31 and HPV-58 [34]. Interestingly, we found that in our population, HPV-18 is the most infrequent (3.72%), and while in our previous study the HPV-39 was highly frequent, HPV-68 revealed to be the most frequent of all after 5 years accumulation of data. In the previous study we showed that HPV-16 predominated in women 30-45 years, which is consistent with worldwide published studies [7,34,35], however now HPV-31 seems to be the predominant in women 30-40 years whereas and HPV-68 in women 45-64 years. Felix *et al.* [36] developed a study to identify the Hr-HPVs that were associated with cervical cancer between 1928 and 2005 in Portugal, showing that in our population the most common HPVs in ICC cases were HPV-16 (58.2%), HPV-18 (9.2%), HPV-33 (6.2%), HPV-45 (4.7%) and HPV-31 (4.4%) [36]. Our data seems to show that there might be a changing n the prevalence of some HPVs, especially those included in the vaccines which might be important for the future. Hence, the strategy of specifically identifying any and all Hr-HPV genotypes in a single sample is, in our viewpoint, clearly advantageous. In addition, data generated by extended genotyping also provides invaluable information concerning the relative frequencies and dynamics of specific Hr-HPV infection in the target population, enabling improved assessment of the actual and future efficacy of vaccination programs and forecast changes in infection patterns.

This is the largest study on Hr-HPV genotyping for Cervical Cancer Screening including a total of 462.401 women over a period of 65 months. In our study, the overall prevalence of Hr-HPVs in women from the Northern region of Portugal was of 12.5%, and the most common Hr-HPVs genotypes are HPV-68, HPV-31, HPV-52, HPV-51 and HPV-16. Age-related Hr-HPV genotype distribution shows that HPV-31 predominated in women 30-40 years whereas HPV-68 predominated in women 45-64 years. For the first time, we correlate the Hr-HPV genotype with cytological evaluation. Cervical cytology was available for 57.103 Hr-HPV positive cases of which 65.68% were NILM, 20.83% ASC-US, 8.86% LSIL, 1.65% HSIL, 2.85% ASC-H, 0.09%

AGC, 0.01% AdC, and 0.01% ICC. NILM was highly frequent in all age-groups, ranging from 62.78% at 35 years old and 77.47% at 64 years old.

Regarding the correlation between the different Hr-HPV genotypes and cytological distribution, we observed that Multiple Hr-HPV infections represented 22.44% of NILM, 33.36% of ASC-US, 44.52% of LSIL, 35.10% of HSIL, 34.85% of ASC-H, 0% of ICC, 22.64% of AGC and 33.33% of AdC. HPV-16/18 infections represented 11.40% of NILM, 16.68% of ASC-US, 15.72% of LSIL, 51.96% of HSIL, 38.04% of ASC-H, 90.00% of ICC, 43.40% of AGC and 66.67% of AdC. Hr-HPVs included in the 9-valent vaccine (HPV-9val) represented 52.13% of NILM, 59.21% of ASC-US, 55.06% of LSIL, 90.14% of HSIL, 83.50% of ASC-H, 100% of ICC, 83.02% of AGC and 77.78% of AdC. In Sum, we observed that the Hr-HPV genotypes present in the 9-valent vaccine are responsible for the large majority of cervical lesions and all cancers, which reinforces the utility of this vaccine in the strategy for cervical cancer elimination in our population.

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FINANTIAL DISCLOSURE

All authors declare that they have no competing financial interests.

LIST OF TABLES

LIST OF FIGURES

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CHAPTER III

CHARACTERIZATION OF VAGINAL MICROBIOME IN CERVICAL SAMPLES FROM THE REGIONAL CERVICAL CANCER SCREENING PROGRAM OF THE NORTHERN REGION OF PORTUGAL

AUTHORS

Andreia Rosário [1,2], Ana Sousa[1], Tatiana Varandas[2], Joana Marinho-Dias[2], Rui Medeiros[1,2,4], Gabriela Martins [2], Paula Monteiro [3], Hugo Sousa[1,2]

AFFILIATIONS

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

[2] Clinical Pathology Service, Clinical and Medical Pathology Department, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center (Porto.CCC), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

[3] Pathological Anatomy Service, Clinical and Medical Pathology Department, Portuguese Oncology Institute of Porto (IPO Porto) / Porto Comprehensive Cancer Center (Porto.CCC), Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

[4] Research Department, Portuguese League Against Cancer (LPCC-NRNorte), Estrada Interior da Circunvalação 6657, 4200-172 Porto, Portugal

CORRESPONDENCE TO

Hugo Sousa, MD, PhD

Clinical Pathology Service, Clinical and Medical Pathology Department

Portuguese Oncology Institute of Porto FG EPE

Rua Dr. António Bernardino Almeida

4200-072 Porto, PORTUGAL

Phone: +351 22 508 4000 (ext 5410)

Fax: +351 22 508 4001

E-mail: hugo.sousa@ipoporto.min-saude.pt

CONFLICT OF INTEREST

All authors declare no conflict of interest.

ABSTRACT

The microbiome of a healthy female genital tract is characterized by the presence few bacteria which suffer changes according to different factors. The vaginal microbiome has emerged as potentially influencing the natural history of HPV infections and their clinical impact. In this study we aimed to characterize the vaginal microbiome and correlate with HPV-status and cervical abnormalities.

We performed a cross-sectional study with randomly selected consecutive samples from the cohort of Hr-HPV positive cases processed between September 2021 to November 2021 in the RCCU. Microbiome analysis was performed using commercially available kits.

We enrolled a total of 807 women with mean age 41.45 ±10.79 years old (median age 41; range 24-64), classified into different groups; 319 (39.5%) NILM, 111 (13.8%) ASC-US, 114 (14.1%) ASC-H, 215 (26.6%) LSIL, 47 (5.8%) HSIL and 1 (0.1%) AdC. Overall, we found positive cases except for *Lymphogranuloma venereum*, being the most frequent ones *U. parvum* (52.5%), *Gardnerella vaginalis* (34.5%), *Atopobium vaginae* (32.6%), *Lactobacillus* spp. (30.7%) and *M. hominis* (23.5%). The risk analysis regarding cytology revealed that infection by *Megasphaera* Tipo 1, *Lactobacillus* spp., *Gardnerella vaginalis*, bacteria associated to bacterial vaginosis, *Atopobium vaginae* and *Mobiluncus* spp. are associated with decreased risk for the development of any cervical abnormality ($p<0.001$; NILM vs any cervical abnormality). Furthermore, similar results were found when analysing the relative risk of development of HSIL or higher (including ASC-H). When analysing the association with HPV we found that *U. urealyticum* and *M. hominis* are associated with increased risk of multiple Hr-HPV infections, while *Lactobacillus* spp., *Gardnerella vaginalis* are associated with decreased risk of multiple Hr-HPV infection. In addition, *Lactobacillus* spp. And *Gardnerella vaginalis* are associated with a protective effect for HPV16/18 infection. Finally, we observed that M *hominis* and *Mobiluncus* spp. are associated with increased risk of infection by one HPV-9val, while *Lactobacillus* spp. and *Gardnerella vaginalis* are associated with a protective effect.

These data showed that overall *Lactobacillus* spp. and *Gardnerella vaginalis* are associated with a protective effect for either HPV-infection of cervical abnormality development while *U. urealyticum*, *M. hominis and Mobiluncus* spp. are more frequently associated with increased risk.

This study provides important data to be used in the clinical approach and management of Hr-HPV positive women.

KEYWORDS: Human Papillomavirus, cervical cancer, vaginal microbiome, screening, genotyping, prevalence, epidemiology.

1. INTRODUCTION

Worldwide, Invasive Cervical Cancer (ICC) is one of the most important cancers in women, being responsible for 569,847 new cases and 311,365 related deaths in 2018, according to Globocan [1]. In Portugal, in 2018 it was estimated a total of 750 new cases and 350 deaths, with an Age-Standardized incidence and mortality rates of 8.9 and 2.8 per 100,000 women, respectively [1].

Since 1970s that Human Papillomavirus (HPV) were identified as the etiological factor of cervical cancer, and since 2005 that The International Agency for Research on Cancer (IARC) consider 14 different HPVs as carcinogens, which are described now as High-Risk HPVs (Hr-HPVs) [2–5]. It is well known that the persistent infection by Hr-HPVs is associated with the development of high-grade cervical lesions that can evolve to ICC [5,6]. There are over 150 different HPV genotypes [3], nevertheless HPV-16 and -18 are responsible for the majority of cervical cancer cases worldwide, and together with HPV-31, -33, -45, -52 and -58 represent over 90% of all cases [6–10]. There are 14 Hr-HPV genotypes (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68) included in the majority of HPV-tests commercialized and its identification may be useful for the development of future HPV vaccines or cervical cancer prevention strategies [11–13].

Hence, there is an increasing interest in analyse the data from full Hr-HPV genotyping to be able to develop better and more accurate strategies for cervical cancer screening. One topic of interest is the role of the microbiome in HPV-induced diseases [14]. The generation of reproducible microbiome archetypes, or community state types (CSTs) [15] most commonly found in patients with cervical lesions were CSTs characterized by *Lactobacillus* depletion, predominance of anaerobic bacteria and dominance of Lactobacillus iners [16-19]. *Sneathia* was significantly enriched in cervical lesions samples in multiple studies, and its presence was associated with alterations in immune mediators [18,20]. A study of the microbiome in cervico-vaginal samples from the Portuguese population, by Jani Silva *et al*, suggests that genital *Ureaplasma urealyticum, U. parvum* and *Mycoplasma hominis* are common in Portuguese women of reproductive age (<30 years old) while *M. genitalium* is rare in the same population [21]. Thus, we can infer the importance of studying the vaginal microbiome and its possible influence on HPV infections.

2. MATERIAL AND METHODS

2.1 STUDY POPULATION

This study was performed using samples from a biological bank of women that participate in the Regional Cervical Cancer Screening Program from the Northern Region of Portugal (RCCU) between 2016 and 2021. The RCCU is an organized screening performed in all women from the North Region of Portugal aged 25–60 years old (with extension up to 64 years of age). All cervical samples from the North region of Portugal are sent to the reference laboratory at the Portugues Oncology Institute of Porto (IPO Porto) and tested for Hr-HPV using the *Anyplex II HPV HR Detection kit* (Seegene, Seoul, Korea) as previously described [22]. This method allows for simultaneous detection and genotyping of 14 Hr-HPV genotypes (-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66 and -68). Cases are processed and reminiscent DNA is stored at -80ºC during at least 1 year until being eliminated.

Hr-HPV positive cases undergo cytopathological examination, performed by dedicated cytopathologists, and are classified the Bethesda Classification (Modified): Negative for Intraepithelial Lesion or Malignancy (NILM); Atypical Squamous Cells of Undetermined Significance (ASC-US); Atypical Squamous Cells that cannot exclude an HSIL (ASC-H); Low-Grade Squamous Intraepithelial Lesions (LSIL); High-Grade Squamous Intraepithelial Lesions (HSIL); Adenocarcinoma (AdC) and ICC.

For methodological convenience, we randomly selected consecutive samples from the cohort of Hr-HPV positive cases processed between September 2021 to November 2021 that underwent cytopathological evaluation, and split in different groups according to cytology: NILM, ASC-US, ASC-H, LSIL and HSIL.
This study was approved by the Institutional Ethical Committee (ref. CES-IPO:146/022).

2.2 MICROBIOME ANALYSIS

Microbiome analysis was performed in the deoxyribonucleic acid (DNA) stored from the selected samples using 3 different commercial kits: *Allplex™* STI Essential Assay Q (Seegene, Seoul, South Korea), *Allplex™* Genital Ulcer Assay (Seegene, Seoul, South Korea) and *Allplex™*

Bacterian Vaginosis Assay (Seegene, Seoul, South Korea). The *Allplex™ STI Essential Assay Q (MH, UU)* enables simultaneous amplification and detection of *C. trachomatis* (CT*), N. gonorrhoeae* (NG), *M. genitalium* (MG), *M. hominis* (MH), *U. urealyticum* (UU), *U. parvum* (UP) and *T. vaginalis* (TV). The *Allplex™ Genital Ulcer Assay* enables simultaneous amplification and detection of *Herpes simplex virus type 1* (HSV-1), *Herpes simplex virus type 2* (HSV-2)*, H. ducreyi* (HD), *Cytomegalovirus* (CMV), *Lymphogranuloma venereum* (LGV, *C. trachomatis* Serovar L), *T. pallidum* (TP) and *Varicella-zoster virus* (VZV). The *Allplex™ Bacterial Vaginosis Assay* amplifies and detects of *Megasphaera* Tipo 1 (Mega1), *Lactobacillus* spp. (Lacto), *Bacteroids fragilis* (BF), *Gardnerella vaginalis* (GV), bacteria associated to bacterial vaginosis (BVAB2), *Atopobium vaginae* (AV) and *Mobiluncus* spp. (MOB).

These kits use a MuDT™ technology which provide multi-C_t (cycle threshold) values in a single fluorescence channel without melting curve analysis. Polymerase Chain Reactions (PCRs) are prepared using 5ul of RNase-free water, 5uL of EM1 (Enzyme Master) and 5ul of specific probes/primers for each Kit. The amplification conditions are performed according to the manufacturer. Reactions are performed in a Real-Time PCR system (CFX96 PCR from Bio-Rad and results are analysed using Seegene Viewer™ (Seegene, Seoul, South Korea) data analysis software.

2.3 STATISTICAL ANALYSIS

The statistical analysis was performed by descriptive statistics (frequencies/prevalence) and the correlation was performed using the computer software IBM SPSS Statistics for Mac, Version 24.0 (Armonk, NY: IBM Corp). Categorical variables were expressed using counts and percentages. Continuous data were presented as mean with standard deviation (±SD) or as median with range and compared using Student's t-test and ANOVA considering a statistical significance of 5% (p<0.05). The Chi-square test was used to assess the association between groups and different categorical variables considering a 5% or less statistically to compute for risk ratio (RR) and 95% confidence intervals (CI).

3. RESULTS

3.1 STUDY POPULATION

We enrolled a total of 807 women with mean age 41.45 ±10.79 years old (median age 41; range 24-64), classified into different groups; 319 (39.5%) NILM, 111 (13.8%) ASC-US, 114 (14.1%) ASC-H, 215 (26.6%) LSIL, 47 (5.8%) HSIL and 1 (0.1%) AdC– **Table 1**. No significant difference in the mean age of the population was found for the majority of groups.

Table 4 - Description of the groups included in the study population.

	Number of cases	Age (mean ± sd), years old	Hr-HPV Multiple Infection (n, %)	HPV-16/-18 (n, %)	HPV-9val (n, %)
NILM	319	43.14 ± 11.29	73 (22.9)	49 (15.4)	183 (57.4)
ASC-US	111	41.64 ± 11.16	49 (44.1)	28 (25.2)	71 (64.0)
ASC-H	114	41.41 ± 9.79	32 (28.1)	44 (38.6)	108 (94.7)
LSIL	215	38.80 ± 10.38	106 (49.3)	41 (19.1)	130 (60.5)
HSIL	47	41.94 ± 8.37	12 (25.5)	25 (53.2)	42 (89.4)
AdC	1	-----	----	1 (100)	1 (100)
TOTAL	807	41.45 ±10.79	272 (33.87%)	188 (23.3%)	535 (66.3)

3.2 VAGINAL MICROBIOME

Overall, we found positive cases for all microorganisms tested except for *Lymphogranuloma venereum* (LGV, *C. trachomatis* Serovar L) – **Table 2**. Regarding the GU kit we found n=39 (5.2%) CMV, n=21 (2.8%) HSV-2, n=3 (0.4%) VZV, n=1 (0.1%) HSV-1, n=1 (0.1%) HD and n=1 (0.1%) TP. In the STI kit we found n=404 (52.5%) UP, n=181 (23.5%) MH, n=125 (16.3%) UU, n=6 (0.8%) NG, n=39 (5.1%) CT, n=20 (2.6%) TV and n=13 (1.7%) MD. As for the BV kit we found n=266 (34.5%) GV, n=252 (32.6%) AV, n=237 (30.7%) Lacto, n=112 (14.5%) Mega1, n=109 (13.5%) BVAB2, n=80 (10.4%) MOB and n=3 (0.4%) BF.

Table 5 - Cytological distribution and correlation with vaginal microbiome.

		Cytology						HPV status		
	n	NILM, n=319	ASC-US, n=103	LSIL, n=215	HSIL, n=47	ASC-H, n=114	AdC, n=1	Hr-HPV Multiple Infection, n=272	HPV-16/-18, n=188	HPV-9val, n=535
GU Kit										
HSV-1	1	-	1	-	-	-	-	1	-	-
HSV-2	21	7	5	3	2	4	-	7	3	14
HD	1	-	1	-	-	-	-	-	-	1
CMV	39	16	5	9	4	5	-	11	9	26
TP	**1**	1	-	-	-	-	-	-	-	1
VZV	**3**	-	3	-	-	-	-	-	-	2
LGV	**0**	-	-	-	-	-	-	-	-	-
STI Kit										
UU	**125**	47	21	35	3	19	-	59	25	90
NG	**6**	2	-	4	-	-	-	1	2	5
MH	**181**	78	25	43	8	27	-	77	43	132
MG	**13**	3	3	5	-	2	-	5	2	10
UP	**404**	173	56	111	14	49	1	149	88	265
CT	**39**	13	9	15	1	1	-	15	12	28
TV	**20**	9	3	5	1	2	-	8	3	16
BV kit										
Mega1	**112**	83	4	11	4	10	-	33	21	76
Lacto	**237**	147	36	37	5	12	-	67	37	131
BF	**3**	1	1	-	1	-	-	2	2	3
GV	**266**	196	21	29	7	13	-	77	42	161
BVAB2	**109**	78	5	13	3	10	-	35	23	75
AV	**252**	139	42	46	7	18	-	85	52	165
MOB	**80**	58	5	11	2	4	-	31	21	62

Risk analysis (data not shown, based on **Table 2**) revealed that infection by Mega1 ($p<0.001$; RR=0.19; 95% CI 0.12-0.30), Lacto ($p<0.001$; RR=0.29; 95% CI 0.21-0.40), GV ($p<0.001$; RR=0.11; 95% CI 0.08-0.16), BVAB2 ($p<0.001$; RR=0.22; 95% CI 0.14-0.35), AV ($p<0.001$; RR=0.43; 95% CI 0.31-0.58) and MOB ($p<0.001$; RR=0.23; 95% CI 0.14-0.38) are associated with decreased risk for the development of any cervical abnormality (NILM vs any cervical

abnormality). Similar results are found if we combine NILM and ASC-US *versus* any cervical lesion (data not shown).

When analysing the relative risk of development of HSIL or higher (including ASC-H), the analysis showed that UU(p=0.009; RR=0.62; 95% CI 0.43-0.89), CT (p=0.021; RR=0.21; 95% CI 0.05-0.90), Mega1 (p=0.045; RR=0.55; 95% CI 0.30-0.99), Lacto (p<0.001; RR=0.23; 95% CI 0.14-0.40), GV (p<0.001; RR=0.23; 95% CI 0.14-0.39), BVAB2 (p=0.033; RR=0.52; 95% CI 0.28-0.96), AV (p<0.001; RR=0.34; 95% CI 0.22-0.55) and MOB (p=0.004; RR=0.30; 95% CI 0.14-0.72) are associated with decreased risk.

When analysing the association of vaginal microbiome with Hr-HPV status (data not shown, based on **Table 2**), we found that UU (p<0.001; RR=1.94; 95% CI 1.31-2.86), MH (p=0.005; RR=1.63; 95% CI 1.16-2.30) are associated with increased risk of multiple Hr-HPV infections, while Lacto (p=0.026; RR=0.68; 95% CI 0.49-0.96), GV (p=0.036; RR=0.71; 95% CI 0.51-0.98) are associated with decreased risk of multiple Hr-HPV infection. When analysing the risk associated with HPV-16/-18 infection, we found that Lacto (p<0.001; RR=0.51; 95% CI 0.34-0.76), GV (p<0.001; RR=0.50; 95% CI 0.34-0.73) are associated with a protective effect. In addition, we found that MH (p=0.028; RR=1.51; 95% CI 1.04-2.18) and MOB (p=0.025; RR=1.85; 95% CI 1.07-3.20) was associated with increased risk of infection by one HPV-9val, while Lacto (p<0.001; RR=0.50; 95% CI 0.36-0.68), GV (p=0.014; RR=0.68; 95% CI 0.50-0.92) are associated with a protective effect.

4. DISCUSSION

The vaginal microbiome is composed by a multiple microbial species, including genital mycoplasmas and ureaplasmas [23,24]. Studies on the prevalence of vaginal microbiome are limited, which could be related to the controversy of infection or colonisation by these microorganisms [25].

To the best of our knowledge, we report for the first time the prevalence of such a variety of microorganisms in samples from women obtained in the scope of RCCU and tested their association with cytological and Hr-HPV status. The most prevalent microorganisms founded in this study are UP with 404 (52.5%) cases, GV with 266 (34.5%) cases, AV with 252 (32.6%) cases, 237 (30.7%) cases and MH with 181 (23.5%) cases. Overall, the prevalence of UP and UU is in agreement with reports of other authors [26-29].

The microbial vaginal flora changes significantly throughout the different phases of women's lives (including birth, infancy, teenage, reproductive age and menopause) which is associated with levels of oestrogen. The oestrogen cycle stimulates the thickness of the vaginal epithelium in women of childbearing age, and the lower vaginal pH (< 4.5) is sustained by the vaginal microbiota that is mainly composed by Lactobacillus [30]. The vaginal dysbiosis occasionally causes symptomatic conditions [31], however, the well-studied and commonest clinical condition typified by vaginal dysbiosis is BV, that is linked to barely any vaginal inflammation [31]. Many studies have shown that BV is followed by alterations in the microenvironment of the vagina, reduced lactate, high vaginal pH and more abundant other bacterial populations (GV, MH, MOB and UU) [30, 32-35].

In our study, we observed some differences in the microbiome profile and the cytological abnormalities patterns or Hr-HPv distribution. The risk analysis revealed that infection by Mega1, Lacto, GV, BVAB2, AV and MOB are associated with decreased risk for the development of any cervical abnormality (NILM vs any cervical abnormality). Similar results are found if we combine NILM and ASC-US *versus* any cervical lesion. In addition, the analysis showed that UP, CT, Mega1, Lacto, GV, BVAB2, AV *and MOB* are associated with decreased

relative risk of development of HSIL or higher (including ASC-H). When analysing the association of vaginal microbiome with Hr-HPV status, we found that UU and MH are associated with increased risk of multiple Hr-HPV infections, while Lacto and GV are associated with decreased risk. Furthermore, we found that Lacto and GV are associated with a protective effect of HPV-16/-18 infection. The data also showed that MH and MOB was associated with increased risk of infection by HPV-9val, while Lacto and GV are associated with a protective effect.

In conclusion, we saw that UU and MH are associated with increased risk of multiple Hr-HPV infections, while Lacto and GV are associated with decreased risk. Otherwise, our results demonstrated that Lacto and GV are associated with a protective effect of HPV-16/-18 infections. The data also showed that MH and MOB was associated with increased risk of infection by HPV-9val, while Lacto and GV are associated with a protective effect.

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FINANTIAL DISCLOSURE

All authors declare that they have no competing financial interests.

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CHAPTER IV

DISCUSSION

The new strategies for cervical cancer screening support the use of HPV test as primary test (higher sensitivity) followed by triage of HPV-positive results with cytology (higher specificity) [19–22]. Literature describes that HPV DNA testing is more sensitive for identifying women with CIN 2+ compared with cytology, despite having a lower specificity [16,23–25]. Indeed, studies shown that in women aged 30 to 69 years the sensitivity of HPV test is around 95% compared with 55% for cytology [20].

Portugal has a high-incidence of cervical cancer when compared with the majority of European countries and therefore Portuguese governments have started with vaccination and more effective screening strategies since 2008. Since September 2017, the Portuguese Ministry of Health has decided that cervical cancer screening should be performed using HPV test as the primary screening method. Hr-HPV full genotyping has been used more often since it is considered to provide additional epidemiological data [26–28]. In this study we report the prevalence of Hr-HPV genotypes detected in samples from women attending the cervical cancer screening from the Northern region of Portugal over a period of 5 ears and combine the information regarding the cytological classification of the Hr-HPV positive cases [25,28,29].

Our results report an overall prevalence of Hr-HPV of 12.5% which is significantly different from the reported value (19.4%) in the CLEOPATRE study from Pista *et al.* [30] and the reported (17.8%) in the Catalan Institute of Oncology (ICO)/IARC Information Centre on HPV and Cancer [31]. Nevertheless, this prevalence is similar to the expected prevalence in the majority of European countries [31]. The data we have first reported between August 2016 and December 2017 discloses a prevalence of 10.2% ranging from a maximum of 17.1% at age 25 to a minimum of 6.2% at age 64, and with simultaneous infections by two or more Hr-HPVs to represent 25.7% (31.0% at age of 25 to 16.5% at age of 64) [32]. Currently, by analysing 5 years of data in RCCU, we observe different results: the Hr-HPV prevalence is 12.5% varying according to age from a maximum of 20.8% at age 25 to a minimum of 8.3% at age 64. Despite the changes, data corroborate published evidence that HPV infections are extremely more frequent in young

women and tend to become much lower after the age of 45 [33]. Furthermore, actually the Hr-HPV multiple infections rate is 27.14% and it is important to analyse the potential impact of this data since it is unclear what is the biological behaviour of multiple infections and its outcome.

Regarding the HPV genotypes, in our previous study we found that the most prevalent were HPV-16 (17.5%), HPV-39 (16.7%), HPV-31 (15.0%), HPV-68 (13.2%), HPV-52 (10.7%) and HPV-51 (10.6%) [32] and now the 5 most common Hr-HPV genotypes found in our population were HPV-68 (16.09%), HPV-31 (15.3%), HPV-52 (13.33%), HPV-51 (12.96%) and HPV-16 (11.06%). Worldwide, the five most prevalent types are HPV-16, followed by HPV-18, HPV-52, HPV-31 and HPV-58 [34]. Interestingly, we found that in our population, HPV-18 is the most infrequent (3.72%), and while in our previous study the HPV-39 was highly frequent, HPV-68 revealed to be the most frequent of all after 5 years accumulation of data. In the previous study we showed that HPV-16 predominated in women 30-45 years, which is consistent with worldwide published studies [7,34,35], however now HPV-31 seems to be the predominant in women 30-40 years whereas and HPV-68 in women 45-64 years. Felix *et al.* [36] developed a study to identify the Hr-HPVs that were associated with cervical cancer between 1928 and 2005 in Portugal, showing that in our population the most common HPVs in ICC cases were HPV-16 (58.2%), HPV-18 (9.2%), HPV-33 (6.2%), HPV-45 (4.7%) and HPV-31 (4.4%) [36]. Our data seems to show that there might be a changing n the prevalence of some HPVs, especially those included in the vaccines which might be important for the future. Hence, the strategy of specifically identifying any and all Hr-HPV genotypes in a single sample is, in our viewpoint, clearly advantageous. In addition, data generated by extended genotyping also provides invaluable information concerning the relative frequencies and dynamics of specific Hr-HPV infection in the target population, enabling improved assessment of the actual and future efficacy of vaccination programs and forecast changes in infection patterns.

This is the largest study on Hr-HPV genotyping for Cervical Cancer Screening including a total of 462.401 women over a period of 65 months. In our study, the overall prevalence of Hr-HPVs in women from the Northern region of Portugal was of 12.5%, and the most common Hr-HPVs genotypes are HPV-68, HPV-31,

HPV-52, HPV-51 and HPV-16. Age-related Hr-HPV genotype distribution shows that HPV-31 predominated in women 30-40 years whereas HPV-68 predominated in women 45-64 years. For the first time, we correlate the Hr-HPV genotype with cytological evaluation. Cervical cytology was available for 57.103 Hr-HPV positive cases of which 65.68% were NILM, 20.83% ASC-US, 8.86% LSIL, 1.65% HSIL, 2.85% ASC-H, 0.09% AGC, 0.01% AdC, and 0.01% ICC. NILM was highly frequent in all age-groups, ranging from 62.78% at 35 years old and 77.47% at 64 years old.

The vaginal microbiome is composed by a multiple microbial species, including genital mycoplasmas and ureaplasmas [23,24]. Studies on the prevalence of vaginal microbiome are limited, which could be related to the controversy of infection or colonisation by these microorganisms [25].
To the best of our knowledge, we report for the first time the prevalence of such a variety of microorganisms in samples from women obtained in the scope of RCCU and tested their association with cytological and Hr-HPV status. The most prevalent microorganisms founded in this study are UP with 404 (52.5%) cases, GV with 266 (34.5%) cases, AV with 252 (32.6%) cases, 237 (30.7%) cases and MH with 181 (23.5%) cases. Overall, the prevalence of UP and UU is in agreement with reports of other authors [26-29].

The microbial vaginal flora changes significantly throughout the different phases of women's lives (including birth, infancy, teenage, reproductive age and menopause) which is associated with levels of oestrogen. The oestrogen cycle stimulates the thickness of the vaginal epithelium in women of childbearing age, and the lower vaginal pH (< 4.5) is sustained by the vaginal microbiota that is mainly composed by Lactobacillus [30]. The vaginal dysbiosis occasionally causes symptomatic conditions [31], however, the well-studied and commonest clinical condition typified by vaginal dysbiosis is BV, that is linked to barely any vaginal inflammation [31]. Many studies have shown that BV is followed by alterations in the microenvironment of the vagina, reduced lactate, high vaginal pH and more abundant other bacterial populations (GV, MH, MOB and UU) [30, 32-35].

In our study, we observed some differences in the microbiome profile and the cytological abnormalities patterns or Hr-HPv distribution. The risk analysis revealed that infection by Mega1, Lacto, GV, BVAB2, AV and MOB are associated with decreased risk for the development of any cervical abnormality (NILM vs any cervical abnormality). Similar results are found if we combine NILM and ASC-US *versus* any cervical lesion. In addition, the analysis showed that UP, CT, Mega1, Lacto, GV, BVAB2, AV *and MOB* are associated with decreased relative risk of development of HSIL or higher (including ASC-H). When analysing the association of vaginal microbiome with Hr-HPV status, we found that UU and MH are associated with increased risk of multiple Hr-HPV infections, while Lacto and GV are associated with decreased risk. Furthermore, we found that Lacto and GV are associated with a protective effect of HPV-16/-18 infection. The data also showed that MH and MOB was associated with increased risk of infection by HPV-9val, while Lacto and GV are associated with a protective effect.

CHAPTER V

CONCLUSIONS AND FUTURE WORK

Regarding the correlation between the different Hr-HPV genotypes and cytological distribution, we observed that Multiple Hr-HPV infections represented 22.44% of NILM, 33.36% of ASC-US, 44.52% of LSIL, 35.10% of HSIL, 34.85% of ASC-H, 0% of ICC, 22.64% of AGC and 33.33% of AdC. HPV-16/18 infections represented 11.40% of NILM, 16.68% of ASC-US, 15.72% of LSIL, 51.96% of HSIL, 38.04% of ASC-H, 90.00% of ICC, 43.40% of AGC and 66.67% of AdC. Hr-HPVs included in the 9-valent vaccine (HPV-9val) represented 52.13% of NILM, 59.21% of ASC-US, 55.06% of LSIL, 90.14% of HSIL, 83.50% of ASC-H, 100% of ICC, 83.02% of AGC and 77.78% of AdC. In Sum, we observed that the Hr-HPV genotypes present in the 9-valent vaccine are responsible for the large majority of cervical lesions and all cancers, which reinforces the utility of this vaccine in the strategy for cervical cancer elimination in our population.

In conclusion, we saw that UU and MH are associated with increased risk of multiple Hr-HPV infections, while Lacto and GV are associated with decreased risk. Otherwise, our results demonstrated that Lacto and GV are associated with a protective effect of HPV-16/-18 infections. The data also showed that MH and MOB was associated with increased risk of infection by HPV-9val, while Lacto and GV are associated with a protective effect.

For future work, we want to create an algorithm based on the risk associated with different HPVs, complemented with information from vaginal microbial profiles.

It would be interesting to do a clinical study in women with HPV without lesion, test the strengthening of the vaginal microbial flora and evaluate the rate of HPV clearance.

CHAPTER VI

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