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Comparison of ELISA protocols measuring HPV16 IgG antibodies and evaluation of the association between HPV16 seropositivity and HPV DNA detection

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Résumé

Bien que les infections cervicales au VPH soient très courantes, la séroconversion ne se produit pas toujours. Nous avons comparé deux protocoles basés sur deux dilutions sériques pour mesurer la séroréactivité du papillomavirus humain (VPH) de type 16 et avons étudié si la présence de l'ADN du VPH était associé à la séropositivité au VPH16. Nous avons également évalué si l'association était influencée par la co-infection avec d'autres types de VPH et par la charge virale.

Les données utilisées proviennent de femmes brésiliennes qui ont participées à l'étude de cohorte Ludwig-McGill portant sur l'histoire naturelle de l'infection du col de l'utérus par le VPH. Les protocoles de sérologie étaient basés sur des particules pseudo-virales (VLP) composés par les protéines L1 ou L1 et L2 qui sont, respectivement, les protéines principale et secondaire de la capside virale. Deux dilutions sériques ont aussi été utilisées, soient : 1:10 et 1:50. La séroréactivité au VPH16 a été exprimée en rapports d'absorbance normalisé (NAR). Le génotypage de l'ADN du VPH et la charge virale ont été évalués par des méthodes basées sur la PCR. La corrélation et la concordance entre les dilutions de chaque protocole (VLP L1 et L1+L2) ont été évaluées par la corrélation de Pearson (r) et la méthode de Bland-Altman, respectivement. La performance des différents protocoles a été comparée à l'aide de courbe ROC (*receiver operating characteristic)* en utilisant la présence de l'ADN de VPH16 comme étalon-or. La régression linéaire a été utilisée pour analyser l'association entre la séropositivité au VPH16 et la détection de l'ADN du VPH avec les deux protocoles. La présence de l'ADN du VPH a été analysée en fonction (1) des types spécifiques de VPH plus ou moins apparentés

au VPH16 et (2) l'infection VPH16 détectée seule ou en co-infection avec d'autres types de VPH.

Les modèles de régression linéaire présentés ci-haut ont aussi été utilisées sur l'ensemble de la cohorte testée avec le protocole VLP L1+L2 et dilution sérique 1:10. L'impact de l'âge en tant que facteur de confusion potentiel ou modificateur d'effet a été analysé dans ce modèle. Finalement, l'association entre la charge virale de VPH16 et la séroréactivité a été analysée à l'aide de la corrélation de Pearson.

L'ampleur des différences de la moyenne des log_{10}-NAR et les écart-types entre les dilutions sériques observées pour chaque protocole (VLP L1 et L1+L2) étaient, respectivement, -0,081 (0,123) et -0,026 (0,150) unités logarithmiques. Les NARs obtenus par les dilutions sériques utilisées (1:10 et 1:50) pour chaque protocole étaient fortement corrélés (r = 0,87 vs. 0,94, respectivement). Cependant, l'utilisation de VLP L1+L2 a augmenté la performance du test à détecter les anticorps IgG anti-VPH16 en particulier avec la dilution sérique 1:10 [l'aire sous la courbe ROC la plus élevée (IC 95%) = 0,7330 (0,6465 – 0,8495)]. Les modèles de régression ont montré que la séroréactivité au VPH16 n'étaient qu'associée à la présence de l'ADN du VPH16 et non pas aux autres types. Par exemple, les analyses avec le protocole VLP L1+L2 et la dilution sérique 1:10 ont montré que la séroréactivité au VPH16 était associée à la présence de l'ADN du VPH16, β (IC 95%) = 0,24 (0,14 – 0,34), et non pas aux VPH31 ou 35, β (IC 95%) = 0.03 (-0,19 – 0,25), ou VPH52, 67, 33 ou 58, β (IC 95%) = 0,15 (-0,04 – 0,34), comparativement aux femmes infectées par tout autre type de VPH ou négative.

Les analyses sur la cohorte entière avec le même protocole ont aussi montré que l'association entre la séroréactivité et l'ADN du VPH16 était similaire quand l'infection était présente seule ou en co-infection, β (IC 95%) = 0,14 (0,07 – 0,21) et β (IC 95%) = 0,11 (0,01 – 0,21), respectivement, comparativement à celles infectées par tout autre type de VPH ou négative. L'âge n'a pas été un facteur de confusion important et n'a pas été un modificateur d'effet dans l'analyse de l'ensemble de la cohorte. La charge virale du VPH16 n'a pas été corrélée avec la séroréactivité du VPH16, r (95% IC) = -0,04 (-0,34 – 0,27); β (IC 95%) = -0,01 (-0,08 – 0,06). En conclusion, le protocole le plus fortement corrélé avec l'ADN du VPH-16 a été celui avec le VLP L1+L2 et la dilution sérique 1:10. Seule la présence de l'ADN du HPV16 a été associée à la séropositivité au HPV16 (pas d'autre type de HPV), et elle n'a pas été influencée par la co-infection ou la charge virale.

Mots-clés : séroréactivité au VPH16, ADN de VPH, particules pseudo-virales, test immuno-enzymatique, anticorps IgG

Abstract

Although cervical HPV infections are very common, seroconversion does not always occur. We compared two protocols based on two serum dilutions to measure human papillomavirus (HPV) type 16 seroreactivity and investigated if HPV DNA positivity was associated with HPV16 seropositivity. We also assessed if the association was influenced by co-infection with other HPV types and viral load.

The data used are from Brazilian women participating in the Ludwig-McGill cohort study on the natural history of cervical HPV infection. The serology protocols were based on virus-like particles (VLPs) composed by the L1 or L1 and L2 proteins which are, respectively, the major and minor viral capsid proteins. Two serum dilutions were used: 1:10 and 1:50. HPV16 seroreactivity was expressed as normalized absorbance ratio (NAR). HPV DNA genotyping and viral load were evaluated by PCR-based methods. Correlation and agreement between serum dilutions of each protocol (L1 and L1+L2 VLP) were assessed by Pearson's correlation (r) and Bland-Altman method, respectively. The performance of the different protocols was compared using the receiver operating characteristic (ROC) curve using the presence of HPV16 DNA as the gold standard. Linear regression was used to analyze the association between HPV16 seropositivity and the detection of HPV DNA infection with both protocols. The presence of HPV DNA was analyzed based on (1) specific HPV types more or less related to HPV16 and (2) HPV16 infection detected alone or in co-infection with other HPV types.

The linear regression models presented above were also used on the entire cohort tested with VLP L1+L2 and serum dilution 1:10. The impact of age as a potential confounding

factor or effect modifier was analyzed in this model. Finally, the association between HPV16 viral load and seroreactivity was analyzed using Pearson correlation.

The magnitude of log_{10}-NARs mean differences between serum dilutions and their standard deviations for each protocol (L1 and L1+L2 VLP) were -0,081 (0.123) and -0.026 (0.150) log units, respectively. The NARs obtained by the serum dilutions used (1:10 and 1:50) for each protocol were strongly correlated (r = 0.87 vs. 0.94, respectively). However, the use of L1+L2 VLPs increased the performance of the test to detect HPV16 IgG antibodies, especially with the 1:10 serum dilution [the highest ROC area (95% CI) = 0.7330 (0.6465 – 0.8495)]. The regression models showed that HPV16 seroreactivity was uniquely associated with the presence of HPV16 DNA and not with other HPV types. For example, the analyses with the protocol L1+L2 VLP and serum dilution 1:10 showed that HPV16 seroreactivity was associated with the presence of HPV16 DNA, β (95% CI) = 0.24 (0.14 - 0.34), and not to HPV31 or 35, β (95% CI) = 0.03 (-0.19 - 0.25), or HPV52, 67, 33 or 58, β (95% CI) = 0.15 (-0.04 - 0.34), compared to women infected with any other HPV type or negative.

The analysis of the entire cohort shows that the association between HPV16 seroreactivity and HPV16 DNA infection was similar when the infection was present alone or in co-infection, β (95% CI) = 0.14 (0.07 - 0.21) and β (95% CI) = 0.11 (0.01 - 0.21), respectively, compared to those infected with any other HPV type or negative. Age was not a significant confounder nor an effect modifier in the analysis of the entire cohort. The HPV16 viral load was not correlated with HPV16 seroreactivity, r (95% CI) = -0.04 (-0.34 – 0.27); β (95% CI) = -0.01 (-0.08 – 0.06). In conclusion, the protocol with the higher correlation with HPV 16 positivity was that with the L1+L2 VLP and serum dilution 1:10. Only the presence

of HPV16 DNA was associated with HPV16 seropositivity (no other HPV type), and it was not influenced by co-infection or viral load.

Keywords: HPV16 seroreactivity, HPV DNA infection, virus-like particles, Enzyme-linked immunosorbent assay, IgG antibodies

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List of acronyms

A: Adenine

APC: Antigen presenting cells

bp: Base pair

C: Cytosine

CERES: *Comité d'éthique de la recherche en santé*

CI: Confidence interval

CIN II: Cervical intraepithelial neoplasia of grade II

CIN III: Cervical intraepithelial neoplasia of grade III

cLIA: competitive multiplexed, Luminex based immunoassay

cRIA: competitive radioimmunoassay

dATP: Deoxyadenosine triphosphate

dCTP: Deoxycytidine triphosphate

dGTP: Deoxyguanosine triphosphate

DNA: Deoxyribonucleic acid

dNTP: Nucleoside triphosphate

dTTP: Deoxythymidine triphosphate

E1: HPV Early protein 1

E2: HPV Early protein 2

E4: HPV Early protein 4

E5: HPV Early protein 5

E6: HPV Early protein 6

E7: HPV Early protein 7

ELISA: Enzyme-linked immunosorbent assay

G: Guanine

GST-L1: Gluthathione S-transferase-L1-flag-fusion protein

HC2: Hibrid capture 2

HPV: Human papillomavirus

HR-HPV: High oncogenic risk HPV type

HSIL: High grade squamous intraepithelial lesion

HSV2: Herpes simplex virus type 2

IARC: International Agency for Research on Cancer

IgG: Immunoglobulin G

Inj. C.: Injectable contraceptive

IQR: Interquartile range

KCl: Potassium chloride

L1: HPV Late capsid protein 1

L2: HPV Late capsid protein 2

LCR: Long control region

Log: logarithm

LR-HPV: Low oncogenic risk HPV type

LSIL: Low grade squamous intraepithelial lesion

LS-PCR: Low-stringency PCR

mg/mL: Milligram per millilitre

$MgCl_2$: Magnesium chloride

MHC: Main histocompatibility complex

µL: microlitre

µM: micromole

MNCS: Milk and newborn calf serum

mol/L: number of moles per litre

NAR: Normalized absorbance ration

ng: nanogram

^oC: Degree Celsius

OC: Oral contraceptive

OD: Optical density

OR: Odds ratio

ORF: Open reading frame

p: p value

PaVE: Papillomavirus Episteme

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

pmol: picomole

PRR: Prevalence rate ratio

PV: Papillomaviruses

r: Pearson's correlation

R^2: Coefficient of determination

RCT: Randomized clinical trial

ROC: Receiver operating characteristic

RR: Relative risk

SD: Standard deviation

STI: Sexually transmitted infection

T: Timine

Taq: *Thermus aquaticus*

Th1: T Helper cells type 1

Th2: T Helper cells type 2

Treg: Regulatory T cells

TRIS: Tris(hydroxymethyl)aminomethane

TRIS-HCl: Tris-Hydrochloride

USA: United States of America

VLP: Virus-like particle

List of abbreviations

etc.: Et cetera

vs.: Versus

e.g.: From the Latin, *Exempli gratia* (For example)

i.e.: From the Latin, *Id est* (That is)

To everyone who has the courage to start all over.

Acknowledgements

First, I would like to thank Dr. Helen Trottier, my supervisor, for giving me the opportunity to join her team and for her unconditional support in all aspects of this work. She taught me epidemiology with joy, which I will always remember. I also thank Dr. Eduardo Franco, my co-supervisor, for his mentoring, believing in my talent and making me realize that it is never too late to start all over with passion. I would also like to acknowledge the ongoing support that I have received from previous supervisors: Drs. Luisa Lina Villa and François Coutlée. Their great references allowed me to receive numerous scholarships, including the Canadian Institutes of Health Research Master's Award, thus obtaining the means to pursue my dreams.

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Introduction

Human papillomavirus (HPV) infection is a major public health concern globally. Approximately 10% of worldwide cancers are associated to viral infection and more than half of infection-related cancers in women are attributed to HPV (1).

Cervical cancer is a rare consequence of a common sexually transmitted HPV infection, most frequently with HPV16 (50%), the most significant genotype associated with the development of the disease (2). It is ranked as the fourth most common malignancy in women worldwide and the second most common cancer in women aged 15 to 44 years (3-5). In 2012, the world population of women aged ≥ 15 years who were at risk of developing cervical cancer was 2.7 million (6). About 528,000 new cases of cervical cancer were diagnosed in the same year and over 265,000 people died from it. The estimated cumulative incidence of cervical cancer worldwide is 14 cases per 100,000 women aged ≥ 15 years per year (4). The worldwide incidence increased by 0.6% annually between 1980 and 2010 (6). The estimated worldwide cumulative mortality of cervical cancer is 6.8 cases per 100,000 women aged ≥ 15 years per year (4). About 85% of the global burden occurs in developing regions, where the cumulative incidence and mortality estimations of cervical cancer are 15.7 and 8.3 cases per 100,000 women aged ≥ 15 years per year, respectively. While in more developed regions the statistics are 9.9 and 3.3 cases per 100,000 women aged ≥ 15 years per year, respectively (6, 7).

Most HPV infections are transient and are cleared within 1 or 2 years by the immune system (2, 8). Although not all infected women develop measurable HPV antibodies, about 60-70% seroconvert and retain their antibodies at low-levels in the serum (9-12). The duration of natural immunity and whether it can clear an existing infection or protect against reinfection

and cervical precancerous lesions are still unclear (9, 13-23). HPV16 DNA positive women tend to be more frequently seropositive than HPV DNA-negative women (13, 15, 24-28). Several studies have found a positive association between HPV16 seropositivity and HPV DNA positivity; however, some of them did not reach statistical significance (13, 15, 25, 27-33).

There is no gold-standard method for measuring antibodies to HPV infection (34, 35). Several serological assays measuring a wide range of anti-HPV16 antibodies with different properties are currently available for research purposes only (34, 36, 37). They measure humoral immune response of cumulative exposure to the virus (38). In the absence of efficient methods to harvest native antigens from tissue culture, researchers have used virus-like particles (VLP) to study HPV serology. They are composed by recombinantly expressed HPV capsid proteins which self assemble into VLPs lacking the viral genome (39). They can be composed by the major capsid protein only (L1) or L1 together with L2 protein, the minor capsid protein (40). L2 alone lacks the ability to form VLPs, but it can be incorporated when co-expressed with L1 (41).

The capsid proteins L1 and L2 are codified by the L1 and L2 genes, respectively (2). Sequencing analyzes of these genes have shown that L1 has the most conserved DNA sequence between different papillomaviruses. L2 DNA sequence is less conserved compared to L1 (42). There is no report in the literature evaluating which VLP type is better to detect HPV16 seroreactivity in enzyme-linked immunosorbent assay (ELISA) which is the most common method used for HPV seroepidemiological studies. Little is known if L1+L2 VLPs can be responsible for cross-reactivity between HPV types due to their degree of DNA sequence conservation, and if the performance of the immunoassays can be affected by the

prozone effect, a type of interference resulting in false negatives or inaccurately low results which may be caused by a highly concentrated serum (34, 43).

In the present study, we compared two ELISA protocols (L1 VLP vs. L1+L2 VLP) with two serum dilutions (1:10 and 1:50) to measure HPV16 seroreactivity and investigated if HPV DNA positivity was associated with HPV16 seropositivity. We also assessed if the association was influenced by co-infection with other HPV types and viral load. Seroreactivity in our study was expressed in normalized absorbance ratio (NAR) to minimize the measurement errors due to intra- and inter-assay variability of ELISA assays (27, 30, 44-47). Although NAR is an arbitrary value and unitless, it is an internally standardized measure of seroreactivity (46).

This dissertation is composed of five main chapters. In the first chapter, we present an overview of the biology (viral structure, classification, life cycle, diagnosis and natural history), epidemiology of the papillomaviruses (risk factors of these infections based on DNA tests and questionnaires), and of the host immune response followed by a summary of the viral strategies to avoid it. We also present the viral-like particles and the main serological assays, followed by a review of the determinants of HPV16 seroreactivity (seroepidemiology). We conclude the first chapter presenting the relevance of this study. In chapter 2, we state our objectives, present the Ludwig-McGill cohort study, describe the participants of the study, the methods used to test our samples, and the statistical analyses. In this chapter, we emphasized that the methods used in this work are not currently available in the clinics and public health network. Next, we present the ethical considerations of this study and the author's contributions to the Ludwig-McGill study. Chapter 3 presents the manuscript that will be submitted to "The Journal of Infectious Diseases", containing the main results of this study.

The lab work was supervised by Dr. Luisa Lina Villa, and the statistical analysis by Drs. Helen Trottier and Eduardo Franco. João M.G. Candeias and Patrícia Thomann tested the HPV serology, and Andrea Trevisan tested the viral load and did the statistical analysis. In chapter 4, we present some supplemental results. In chapter 5, we discuss our findings in light of the literature, the limits and strengths of the study, and potential threats to internal and external validity. Finally, we present our conclusions.

Chapter 1. Literature review

1.1. Papillomaviruses

Papillomaviruses comprise a diverse group of viruses that are epitheliotropic, species-specific, and they can infect the skin and mucosa of animals and humans (2, 48). To date, they have been found in fish, reptiles, birds, and mammals (49-53). Considering that these viruses have coevolved with their hosts, they have been an evolutionary success for over 500 million years (54, 55).

1.1.1. Viral structure and classification

Human papillomaviruses belong to the family *Papillomaviridae*, a family of non-enveloped, small, and circular viruses with a double-stranded DNA genome of about 8,000 base pairs. The genome is divided into eight open reading frames (genes) — E1, E2, E4, E5, E6, E7, L1, and L2 — coding for 'early' (E) or 'late' (L) viral functions, and an untranslated long control region (LCR) (2). The structure of their capsid is composed of a virally encoded major coat protein, L1 and a minor coat protein, L2, which will be described afterwards. HPV infections are associated with certain anogenital and oropharyngeal cancers (2, 56). Links between HPV and cervical cancer were first suspected more than 40 years ago (57, 58).

HPV classification is based on the nucleotide sequence of the gene coding for the capsid protein L1 (48, 59). Types belonging to different genera share less than 60% similarity, different species within a genus have identity of DNA sequences between 60 and 70%, a novel genotype has less than 90% similarity to any other type, and identity of DNA sequences

between 98-99% defines a variant of type (48, 59). The family *Papillomaviridae* contains 49 genera (*Papillomavirus* α, β, γ, etc.), each of which is further divided into several species.

The Papillomavirus Episteme (PaVE) is a database of curated papillomavirus genomic sequences updated 4 times a year. The PaVE database was created with the objective to provide clinicians, epidemiologists, and bench scientists with a uniform data source (54). So far, about 350 types of papillomaviruses have been described of which more than 200 can infect humans (54, 60). About 40 human types exhibit tropism for the anogenital tract (1, 59). They are classified into two different groups according to their oncogenic risk. The first group is composed by low-risk types (LR-HPV), mainly represented by HPV6 and 11. They are found in 90% of genital warts and low-grade squamous intraepithelial lesions (LSIL) and rarely found in cancer. The second group is composed by high-risk types (HR-HPV), mainly represented by HPV16 and 18. They are associated with high-grade squamous intraepithelial lesions (HSIL) as well as carcinomas (2, 4, 61). HPV18 is most found in adenocarcinomas (4, 5). All HR-HPV types together account for up to 5% of all human cancers and are the necessary cause of 99.7% of cervical cancer, 90% of anogenital cancer, 40% of penile cancers, and 42–60 % of oropharyngeal carcinomas (5, 62, 63). HPV16 is found in about 50% of all cervical cancer cases, HPV18 in approximately 20%, HPV31, 33, 45, 52, and 58 in about 20%, and about 10% of all cases are caused by other HR-HPV types (4, 64, 65).

The number of new HPV types increases very quickly due to metagenomic sequencing, a high throughput technology for sequencing of biological samples (66). The genus alpha-papillomavirus contain 65 cutaneous and mucosal types as yet (60). Members of the alpha 9 and 7 species have been studied in more detail (67). HPV16 belongs to alpha 9 species together with HPV31, 35, 52, 67, 33, and 58. The first two types are considered more related

to HPV16, since they share a common immediate ancestor in the phylogenetic tree (Figure 1, page 7) (68). According to the International Agency for Research on Cancer (IARC), most of these viruses belong to the group of carcinogenic agents (group 1), except HPV67 which is considered probably carcinogenic (group 2B) (69).

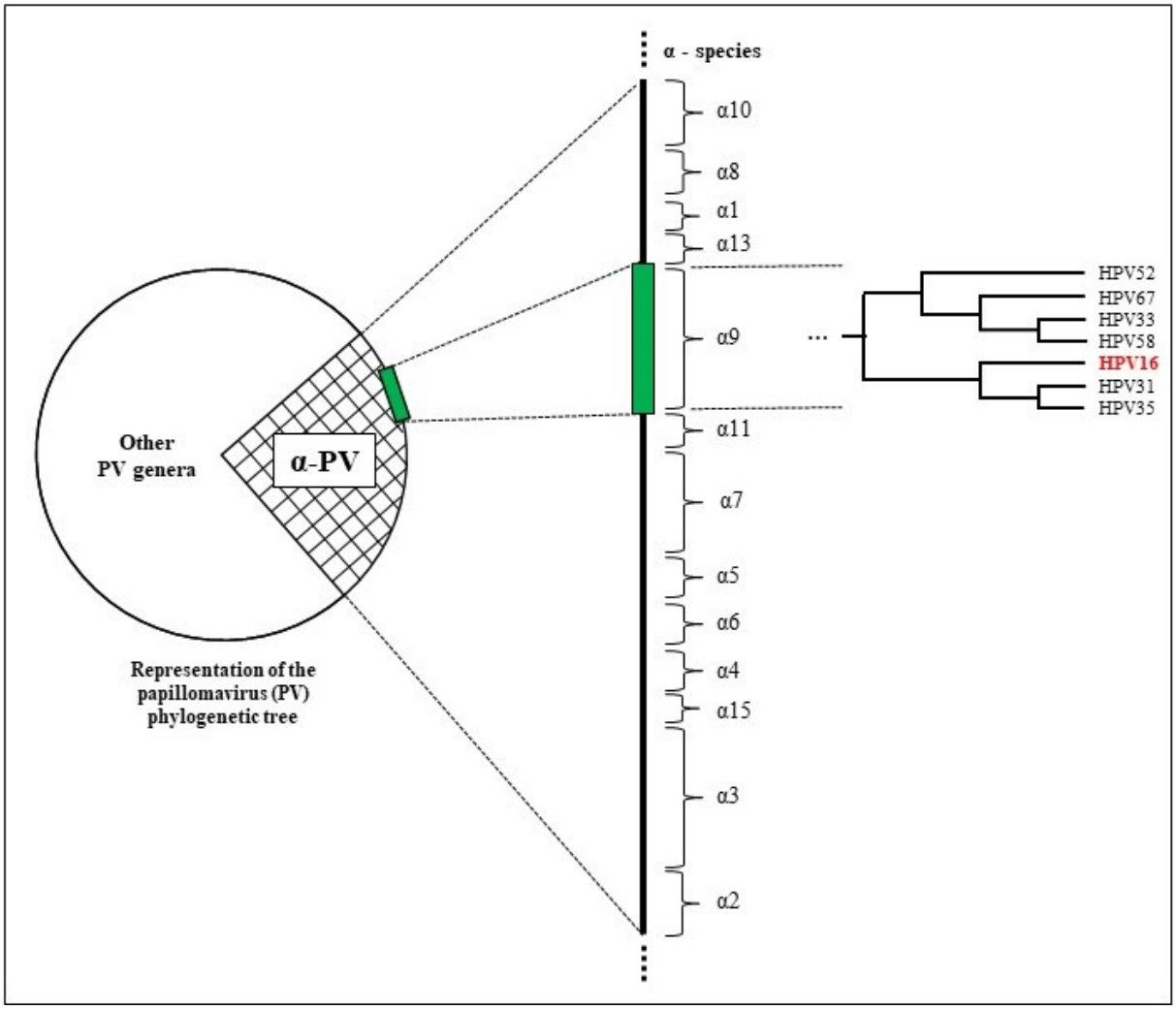

Figure 1: Phylogenetic relatedness of alpha-9 HPV species. Inspired by Schiffman et al., 2011 (68).

1.1.2. HPV life cycle and diagnosis

HPVs infect keratinocytes in the basal layer of the cervical epithelium at low copy numbers as a consequence of microlesions of skin or mucosa. During an infection, HPV genomes are found in the nucleus as episomes, circular extrachromosomal DNA (70). The infected cell divides and spreads laterally increasing the viral load. Some of these cells stops dividing and move into the suprabasal differentiating cell layers. Early viral genes are activated at this point to increase viral genomes to thousands.

Since HPV infection is asymptomatic, it is not possible to predict when it occurs and how soon after infection the presence of the virus can be detected in cervical cells. In clinics, the Pap test is used to look for abnormal cells in the cervix, while the HPV test looks for HPV DNA infection (71, 72). HPV test can find any of the HR-types of HPV that are commonly found in cervical cancer.

Progression to malignancy is frequently associated with loss or viral disruption in the E1/E2 regions and integration into the cellular DNA resulting in the loss of negative feedback control of viral oncogenes (E6 and E7) (73). The moment that integration occurs in the natural history of cervical HPV infections is a controversial issue (74-77). Expression of E6 and E7 oncoproteins is required to maintain the malignant growth of cervical cancer cells by inhibiting cellular tumour suppressors genes (2). The organization of the epithelium changes as the disease progresses (70). In the superficial layers of the epithelium, late viral genes are expressed, and L1 and L2 capsid proteins are formed to encapsidate the viral genomes. Infectious particles are released in the terminally differentiated outer epithelial layer (Figure 2, page 9) (2, 70, 78).

HPVs, especially alpha-species, are very successful infectious agents. They induce chronic infections with no serious sequelae, rarely kill the host and shed large amounts of infectious virus for transmission to other individuals (78).

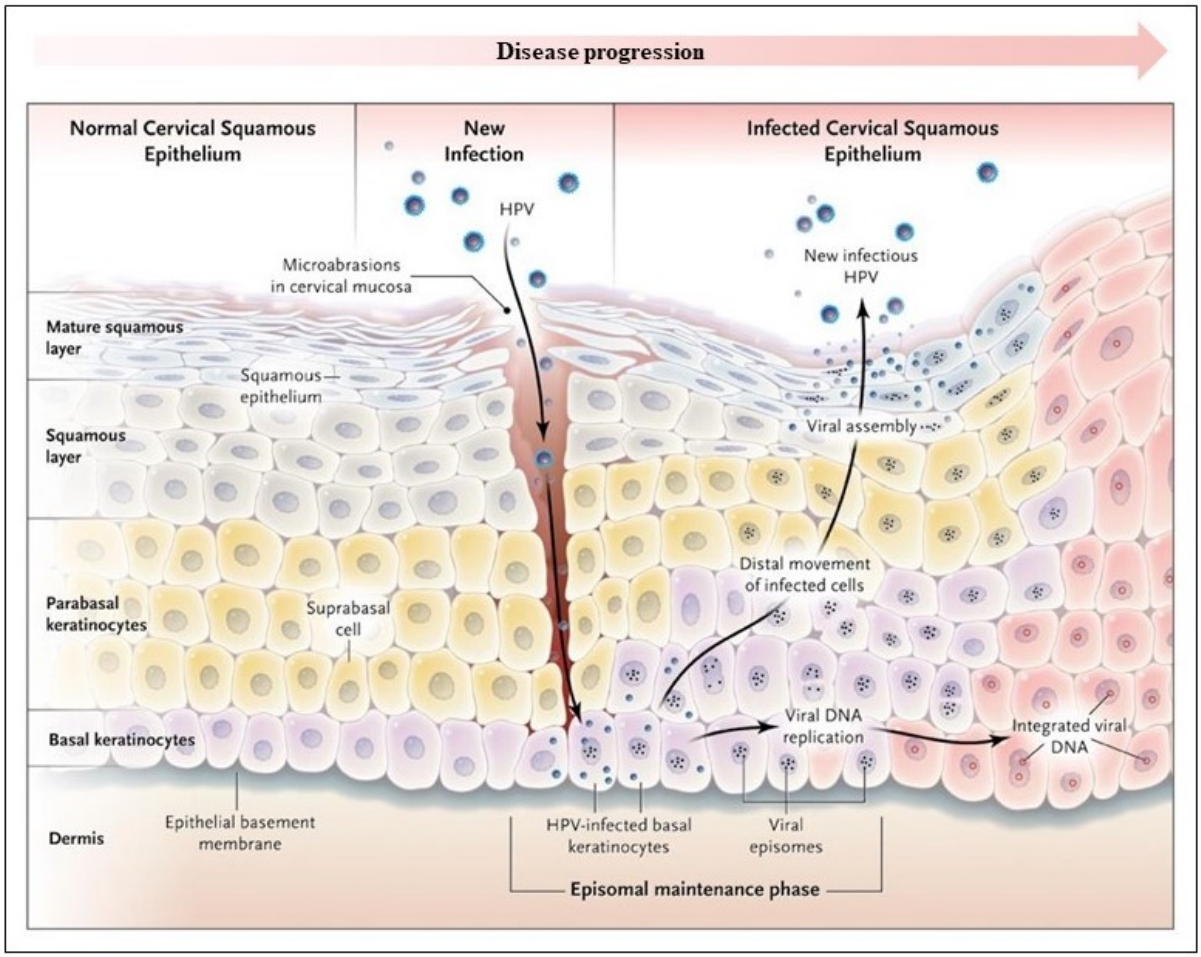

Figure 2: Human papillomavirus life cycle in the squamous epithelium. Reproduced with permission from Kahn, 2009, Copyright Massachusetts Medical Society (70).

1.1.3. Natural history, prevalence and risk factors of HPV infections

HPV is a very common infection acquired via sexual activity (79). More than 80% of women will be infected in their lifetime (80). The incubation period of HPV infections may

last from 3 weeks to more than 8 months (78). Genital warts may occur about 2 to 3 months after infection which end up regressing exponentially in 10-30% of patients within 3 months (78). Infection with HR-HPV types, such as HPV16 and 18 are usually transient and tend to be cleared in 12–18 months due to cell-mediated and humoral immune responses (8, 20, 64, 78, 81, 82). A small number of HPV infections persists, and the pathology may progress to LSIL (10–20%) and HSIL in some cases (20%). If not treated, advanced precancerous lesions may progress to cervical cancer (30-50%) (83-86). Therefore, cervical cancer is a rare consequence of a persistent HPV infection which can be harbored in a latent state for more than 20 years before progressing to cancer (Figure 3, page 10) (87, 88). Definition of HPV persistence varies significantly between studies due to several HPV detection methods available and several lengths of follow-up time (89-91). Consequently, comparison between studies can be challenging (92). In general, persistent infection means two or more HPV-DNA-positive tests, consecutively, in intervals of 4 to 6 months (93).

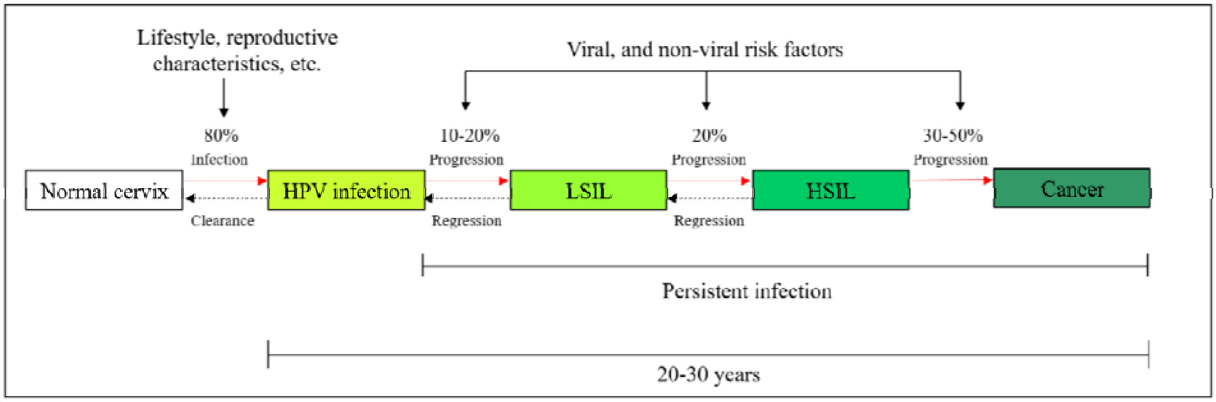

Figure 3: Natural history of HR-HPV infections and the likelihood of progression according to disease severity.

A decade ago, researchers published a meta-analysis based on 78 studies to investigate the age and genotype specific prevalence of HPV infections worldwide (94). Final analysis included 157,879 women with normal cytology. Overall worldwide HPV prevalence was 10.4% (95% CI: 10.2-10.7). Ten years ago, they observed a geographical variation of prevalence estimates by world region: in Africa HPV prevalence was 22.1% (95% CI: 20.9-23.4), Central America and Mexico 20.4% (95% CI: 19.3-21.4), Northern America 11.3% (95% CI: 10.6-12.1), Europe 8.1% (95% CI: 7.8-8.4), and Asia 8.0% (95% CI: 7.5-8.4) (94). Nowadays, the variance in prevalence among different regions of the world has diminished over time probably due to prevention programs (4). Recent data show that HPV prevalence in most regions mentioned above is around 4.0%, except in Asia, where no reduction was observed over the last decade (4). Less developed regions, such as Caribbean with 15.8% (95% CI: 12.2-20.2), South America 12.1% (95% CI: 11.6-12.7), and Eastern Europe 9.7% (95% CI: 9.1-10.4) have the highest HPV prevalence in women with normal cervical cytology worldwide (4).

HPV prevalence was high in women younger than 25 years of age, then decreased in older women in most of the world regions (94). In Africa, the Americas, and Europe, they observed a second peak of HPV prevalence in women aged 45 years or older. Unfortunately, most of age-prevalence data were shown graphically with no precise estimates in the text of the publication. They only provided detailed information on the HPV prevalence in women older than 45 years of age from the Americas. They showed that the overall estimate was higher in Central America (20.4%, 19.3–21.4) and South America (12.3%, 11.2–13.4) than in Northern America (11.3%, 10.6–12.1). HPV16, 18 or both were detected in 32% of the study participants (94, 95). The age-specific bimodal prevalence may be due to a cohort effect

(acquisition of new infections due to new sexual partners), the possibility of reactivation of latent infection or acquired immunity; however, more studies should be done to validate these hypothesis (16, 20, 93, 95, 96).

Overall HPV DNA prevalence grew with increasing severity of cervical disease (4, 94, 97). The more severe the lesion, the greater the probability of detecting the DNA of a HR-HPV type in cells from cervical smears. HPV16 and 18 DNA prevalence according to severity of cervical disease in less developed regions of the world were: 4.4% (95% CI: 4.3-4.5) in normal cytology, 25% (95% CI: 24.1-25.9) in LSIL, 46.6% (95% CI: 45.8-47.4) in HSIL, and 69.5% (95% CI: 68.9-70.1) in cervical cancers (4).

There are some risk factors associated with the acquisition and persistence of HPV infections (98). Some of them can be measured by molecular biology techniques or questionnaires. Cumulative HPV exposure is associated with sexual behaviors, such as number of sexual partners, and concurrent relationships (96, 98, 99). Sexually transmitted infections, such as *Chlamydia trachomatis*, immunodeficiency virus, and bacterial vaginosis have also been found to be predictive of cervical HPV infection risk in epidemiological studies (96, 98, 100-102). Immunodeficiency appears to increase the host susceptibility to infection, since a higher prevalence of genital HPV is observed in immunosuppressed individuals, regardless of the cause of immunosuppression (79, 103).

There are other risk factors which are inconsistently associated to the acquisition of an HPV infection, particularly with respect to reproductive and genital health (98). Micro-abrasions caused during sexual intercourse in a dry and irritated genital tract due to tampon use could increase the susceptibility to HPV infection and decrease the rate of HR-HPV clearance; however, this finding is not supported for all researchers (92, 98, 104, 105). There

is also a lack of consensus regarding smoking, use of oral contraceptive, condom use, and age at first intercourse (96, 99, 102, 106-108). Little is known about the association of frequent vaginal douching and HPV infections (109).

Researchers have also concentrated their efforts to understand the role of risk factors that favor persistence of HPV infection and mediate progression of precancerous lesions to cancer. Some authors have shown that older age and viral factors, such as genotype, molecular variants, and viral load are predictive of persistent HPV infection and progression to cervical cancer (79, 88, 110-115). Smoking, multiparity, long-term use of oral contraceptive, other sexually transmitted infections, and chronic inflammation also seem to increase the risk of persistence, and disease progression (96). Daily consumption of vegetables has also been associated with HPV clearance (92). Besides, it is very common to find co-infection with multiple HPV types in many epidemiological studies (15, 30, 116). However, the role of co-infection on the duration of the infection is not fully understood (101, 117).

1.1.4. An overview of the host immune response

This section is focused on the host immune response and the virus strategies to avoid it. Host immune response against pathogens can be divided in several basic phases that differ depending on the perspective. For the pathogen side, they must find a permissive host environment, successfully initiate infection of target cells and be able to replicate. For the host side, they have to initiate a series of events which includes initial recognition of the pathogen by sentinel host immune cells, establish a innate immune response and trigger an adaptive response to eliminate the pathogen (118). The innate and adaptive immune systems are often described separately; however, they usually act together (119).

1.1.4.1. Innate immunity

Innate immunity is the first line of defense from infection in a non-specific manner by detecting the pathogen and clearing most of microbial assaults (119, 120). It is rapid, does not require prior sensitisation, is not antigen-dependent and has no specific memory (120, 121). In our context, it is an epithelial barrier composed by cells (i.e., phagocytes, some antigen presenting cells, and the effector cells), several cellular antimicrobial products (e.g., cytokines and chemokines), and the complement cascade, a biochemical process that occurs in the blood to help cells of the immune system to eliminate invading pathogens (121).

Briefly, inflammation is the first sign of innate immune response which is triggered by cell injury or death. At this point, the actors of the innate immune system are recruited to solve the infection and kick-start the adaptive immune response if necessary (120).

1.1.4.2. Adaptive immunity

Adaptive immune response is specific and generally lethal to foreign antigens. Antibody-mediated humoral immune response clears free virus particles from body fluids preventing viral reinfection, while cell-mediated immune response kills infected cells and generate immune memory. Both systems are interconnected in some ways with the adaptive immunity becoming prominent several days after the onset of the innate immune response (119, 120).

Antigen-specific immune response is triggered when cells of the innate immune system are stimulated leading to the proliferation and differentiation of cells that compose the adaptive immune system. Lymphocytes T and B are born in the bonne marrow and are the main components of adaptive immune system. Successful cellular and humoral (antibody) host

immune defenses depends on them (120). Next, we provide an overview of the humoral immune response.

1.1.4.2.1. Humoral immune response

Antibodies, also called Immunoglobulins (Ig), are Y-shaped glycoprotein molecules that are produced by plasma cells in response to an antigen. Since different antibodies recognize different antigens, antigen-binding sites are different for different antibodies which are the effector molecules of the humoral immune response (122). Five primary classes of antibodies exist based on the structure of their molecule. They are identified as IgG, IgM, IgA, IgD, and IgE, and are distributed and function differently in the body. IgG has four subclasses (IgG 1 to 4) and is the most frequent (75%) immunoglobulin in the serum. It is versatile because it can carry out all functions performed by all classes of immunoglobulins and provides long term protection (122).

Naïve lymphocytes B are activated when they first encounter an antigen. Only a few native antigens can directly activate B cells and generate plasma cells (120). Low levels of antibodies are produced after natural HPV infection. The response to a second round of infection is often faster than the primary infection because of the activation of memory B and T cells (78). A neutralizing antibody response highly type-specific to L1 is known to effectively prevent HPV infection (123). However, they are unable to kill established HPV-infected cells (62).

HPV vaccination was implemented in several countries in 2007. A systematic review and meta-analysis published at the “Lancet Infectious Diseases” in 2015 showed that HPV16 and 18 infections decreased between the pre-vaccination and post-vaccination periods by 68%

(RR: 0.32, 95% CI: 0.19 – 0.52) and anogenital warts decreased by 61% (RR: 0.39, 95% CI: 0.22 –0.71) in girls 13–19 years of age (124). Significant reductions in HPV 31, 33, and 45 infections were also observed in this age group of girls (RR: 0.72, 95% CI: 0.54 – 0.96) suggesting cross-protection. All these results were observed in high-income countries with female vaccination coverage of at least 50% (124). Vaccination can induce very high concentrations of neutralizing antibodies, at least 2 to 4 log units higher than in natural infections (78).

1.1.4.3. Viral strategies to avoid host immune response

The reason of the successful viral lifestyle is the ability of HR-HPV types to avoid host defence systems (78). The virus replication cycle itself is an immune evasion mechanism that helps the virus to evade the innate immune response and delay activation of adaptive immunity (78, 121).

The HPV life cycle depends on the keratinocyte differentiation program, production of viral particles is time-consuming, there is no cytolysis and no virally induced cell death; consequently, there is no inflammation. All key events occurs in a cell destined for desquamation away from the primary site of immune surveillance, the submucosa (121). During HPV life cycle, there is little or no release of pro-inflammatory cytokines as part of the innate immune response. Cytokines help to trigger the adaptive immune response and are important in the activation and migration of antigen-presenting cells (78). In addition, there is no viremia, and host dendritic cells are exposed to low levels of viral proteins during the natural history of HPV infection (78, 84, 120).

1.1.5. HPV seroepidemiology

In general, natural exposure to a virus results in a protective antibody response; however, seroconversion does not always occur following HPV infections. Only about half of infected women have detectable levels of anti-HPV antibodies in their bloodstream (10, 93). In addition, about half of seropositive women produce neutralizing antibodies (21). For women with incident HPV16 infections, the median time to seroconversion from DNA detection varies from 6-12 months (10, 125). The duration of natural immunity and whether it can protect against cervical precancerous lesions are still unclear (9). Serological assays may identify the individuals who had developed an immune response to previous exposure to HPV and may be protected against reinfection (18, 21, 126). However, some studies have shown that reinfection with the same type is possible suggesting no protection following a previous type-specific infection (20).

Although some researchers have concentrated their efforts to establish an international standard operating procedure for HPV serology, we still do not have a gold-standard method for measuring antibodies to HPV infection (34, 35). Consequently, we have no agreed definition of what level of response indicates effective seroreactivity making comparison between results obtained by different laboratories extremely difficult (34, 78). In addition, HPV serology has several limitations, such as low seroconversion after natural infection, antibody levels may decrease over time, and limited assay sensitivity (10, 13, 127). Due to this variety of technical and biological limitations, HPV serology has not been used in the clinics (45, 128).

In this section we introduce the viral-like particles (VLPs) which are the antigen used in most of the immunoassays and make a brief description of the main serological assays

available to measure antibody titers to HPV infection for research purposes. Finally, we present an overview of the most important findings in the literature about the determinants of HPV16 seroreactivity highlighting the association between HPV16 seroreactivity and HPV DNA positivity.

1.1.5.1. Virus-like particles (VLP): antigens for serological assays

The L1 and L2, major and minor viral capsid proteins, respectively, are assembled late in the HPV life cycle to compose the icosahedral capsid shell which has the function to protect the viral genome (129-132).

In the absence of efficient methods to harvest native antigens from tissue culture, serologic detection of HPV has used virus-like particles (VLP) (39, 45, 131, 133-135). They are non-infectious papillomavirus particles without the viral genome. VLPs display conformational and type-specific epitopes which are the part of an antigen molecule to which an antibody attaches itself. They are structurally similar to authentic virions, term used to designate viral particles outside living cells (39, 135). VLPs are produced in a variety of recombinant expression systems and are highly immunogenic inducing potent antibody responses due to their ability to activate both innate and adaptive immune responses (131, 133-139).

L1 protein can self-assemble to form empty VLPs that are the basis of the licensed HPV vaccines (41, 123, 131). L2 does not form VLPs, but it can be incorporated when co-expressed with L1 (40, 133). L1 has a highly conserved DNA sequence, and L2 is less-well conserved among different HPV types. Addition of L2 in the composition of VLPs can

possibly induce broader protection through cross-neutralizing antibodies, even across species (40, 62, 131).

1.1.5.2. Main serological assays

Serological assays confer an advantage over DNA methods because it is a single outcome that can represent infection from multiple anatomic sites (140, 141). They also can be used as an indicator of cumulative infection exposure to predict the risk of developing cancer and their precursor lesions, reinfection, reactivation, and clearance of infections. (14, 19, 30, 142, 143).

Several serological assays measuring a wide range of anti-HPV16 antibodies with different properties are currently available for research purposes (34, 36). The first assay developed for measuring HPV antibody titers was the athymic mouse xenograft system (144). Due to technical difficulties in testing a large number of sera using this protocol, several complementary assays have been developed (18). Each assay provides only a partial characterization of immune status. They differ quantitatively (i.e., throughput and detection range) and qualitatively (i.e., if they detect polyclonal antibodies which may be indicative of prior exposure or neutralizing antibodies which is indicative of immune protection). Because of that, comparison of seroprevalence across assays is not possible (18, 34, 121)

Several immunoassays have been developed during the last decades, such as the type-specific competitive radioimmunoassay (cRIA) and the pseudovirion-based neutralization assay. Both methods are labor intensive and only measure neutralizing antibodies (145, 146). The last generation of methods have used the Luminex technology to measure neutralizing or total IgG antibodies to VLP (competitive multiplexed, Luminex-based immunoassay, cLIA) or

to glutathione S-transferase-L1-flag-fusion proteins (GST-L1) (147-150). Luminex is a robust, sensitive, and high-throughput serological platform that can be used to measure antibodies to several HPV genotypes at the same time (151). Nevertheless, it is expensive and depends on monoclonal antibodies which specifically bind to only one epitope of the antigen to perform.

The most common serologic assay for HPV is the enzyme-linked immunosorbent immunoassay (ELISA) (152). It is type-specific, but it cannot differentiate between neutralizing and non-neutralizing antibodies. In fact, it measures antibodies to HPV VLPs that were secreted by different B cell clones within the body. So, ELISA measures a polyclonal response. Technically, it means that these antibodies can bind to different epitopes on the same antigen.

In ELISA protocols, antibody measurements have relied on determining VLP optical density (OD) values for serum samples and comparing them against negative and positive controls to detect HPV seroreactivity. However, OD values are prone to measurement errors due to intra- and inter-assay variability originated from daily variations in reagent batches and technical performance (e.g., pipetting, instrument readings, etc.) (46). Our team has proposed the use of normalized absorbance ratio (NAR) to circumvent these technical problems that can affect the validity of seroreactivity (27, 30, 44-47). NARs are calculated by dividing the mean blank-subtracted optical densities (OD) by the equivalent value of the control serum pool included in the same plate in triplicate. Although NAR is an arbitrary value and unitless, it is an internally standardized measure of seroreactivity (46).

Therefore, we are facing a unique opportunity to evaluate which VLP type can better capture the association between naturally acquired HPV16 seropositivity and HPV DNA

positivity using an optimized ELISA, and to investigate if L1+L2 VLPs can be responsible for cross-reactivity between HPV types.

1.1.5.3. Determinants of HPV16 seroreactivity

A review on HPV serology including 117 studies from several world regions has been published (153). Participants were women and men from several hours to over 90 years of age. Serological antibodies were detected with ELISA (78%), cLIA (12%), and other available methods (10%). HPV16 seropositivity was more prevalent in women than in men and peaked around ages 25-40 years in women. Some studies have reported that seroprevalence peaked twice in women. A possible explanation for the second peak at older ages (>50 years) is a reinfection or reactivation of a latent infection maybe by reduction of immune surveillance with increasing age followed by increasing viral load, and antibody induction (26). In young women from 9-26-year-old, HPV16 seroprevalence ranged from 0-31% in North America, 21-30% in Africa, 0-23% in Asia/Australia, 0-33% in Europe, and 13-43% in Central and South America (153).

To better understand the humoral immune response against HPV infections, several researchers from all over the world have identified the determinants of HPV16 seroreactivity. Sexual behavior seems to play an important role in the acquisition of HPV antibodies, particularly, the increased number of lifetime sexual partners (24, 27, 29, 30, 33, 38, 108, 154-161). The exact number of sexual partners that increase the likelihood of seroconversion varies from study to study and depends particularly on the presence of HPV DNA infection among partners. In the Ludwig-McGill cohort study the odds ratio of HPV16 seroreactivity at baseline for women who reported having had more than four lifetime sexual partners were

elevated >2.5-fold compared to women who reported 0-1 partner during their entire life (OR: 2.56, 95% CI: 1.97-3.53) in the analysis adjusted for age and HPV16 DNA positivity (30).

Other determinants of HPV16 seroreactivity were identified, such as smoking, marital status, seropositivity for HPV18, history of sexually transmitted disease other than HPV, hormonal contraceptive use, parity, frequency of sex, years since sexual debut, and high HPV16 viral load (24, 30, 108, 156-159). Age, age at first intercourse, stage of the disease, and cytologic diagnosis are still controversial determinants of HPV16 seroreactivity (24-30, 33, 38, 154, 156-158). However, all these factors might be related to HPV infection only. It is difficult to understand what is related to HPV DNA infection from what is related specifically to seroconversion. The role of potential confounders in the association between HPV16 seropositivity and HPV DNA positivity will be discussed in depth later.

Both cross-sectional and longitudinal study designs have reported the correlation between HPV16 DNA positivity and HPV16 seropositivity (13, 15, 25, 27-33). Based on inclusion and exclusion criteria which are shown in the appendix I, we prepared a summary table of the literature regarding this subject (Appendix II). In brief, all studies mentioned above have found a positive association between HPV16 seropositivity and HPV16 DNA positivity independently of any other factor (e.g., age, number of lifetime sexual partners, etc.). Three of them did not report statistically significant results for the association (29, 31, 32). Only one study presented the results adjusted for age and lifetime number of sexual partners ($p=0.046$) (25). HPV16 DNA positive women tended to be more frequently seropositive than HPV DNA-negative women (13, 15, 24-28). Only two studies found that HPV16 DNA positive women were less seropositive than HPV DNA-negative women which is probably due to their small sample size (31, 32). German researchers have reported two

peaks of HPV seroprevalence according to the age of the participants (26). The first peak is in young adult women (15–34 years) and the second in women older than 45 years old.

The partial analysis of the Finnish family HPV study conducted at Turku, Finland, and designed to evaluate dynamics of HPV infections within families used the Luminex technology with GST-L1 proteins as antigen to detect the antibody levels (150, 162). Authors reported no concordance between cervical DNA detection and co-existent seropositivity even in samples taken 12 months apart, but it showed that women who cleared their cervical HPV16 DNA infection had the highest HPV16 antibody levels, whereas those who acquired incident HPV16 DNA infections had the lowest antibody levels.

1.2. Relevance of the study

Cervical cancer is an important public health problem worldwide. There is no cervical cancer without an HPV infection. From a public health perspective, the government depends on seroconversion results obtained by standardized serological methods to decide the cost-effectiveness of vaccination. This study aims to increase the validity and precision of a serological instrument and; therefore, should contribute directly to the quality and reliability of the decisions from public health institutions.

From a clinical perspective, understanding why some women can produce antibodies after having naturally acquired an HPV infection and others not, particularly infection with HPV16, the most prevalent genotype, can help clinicians to drive personalised and more effective treatments.

Based on the assumption that both clinical and public health professionals depend on a highly performing serological instrument for decision-making, it is important to consider

every detail concerning the methodology used to measure HPV antibodies. An accurate instrument should be valid and precise, which is essential to compare epidemiological studies and ultimately use the findings for the benefit of the population. The lack of precision (whether there is or not dispersion in measurements) and validity (whether the estimation is near or not the true value) in measurements can produce random errors and bias. The epidemiological objective of this study is the analysis of the baseline data of a cohort of women tested by two ELISA protocols performed with two VLP types using two serum dilutions. We sought to identify which of them better capture the association between HPV16 seropositivity and HPV DNA positivity, that is which combination of conditions was the most accurate and precise to measure HPV seropositivity. Although comparisons between serological assays have been done, particularly to measure antibody responses after HPV vaccination, studies comparing L1 and L1+L2 VLP ELISA protocols to measure humoral immune response to naturally acquired HPV infection are lacking in the literature (34, 39, 163, 164).

This study provides data to increase the performance of serological methods with the potential to be used in clinics and public health decision-making in the future. A standardized method is crucial to validate the effect of the HPV vaccine in contrast to naturally acquired immunity. Our findings further our understanding of the natural history of HPV infections, provide us knowledge about the main determinant of HPV16 seroreactivity (HPV DNA positivity), and allow epidemiological researchers to design new epidemiological studies with the objective to answer remaining questions about this issue.

Chapter 2. Methodology

2.1. Objectives

The aims of this study were to compare two protocols (L1 only vs. L1+L2 VLPs) based on two serum dilutions (1:10 and 1:50) to measure HPV16 seroreactivity, to investigate whether HPV DNA positivity was associated with HPV16 seropositivity and to verify if the association was influenced by co-infection with other HPV types and viral load.

2.2. The Ludwig-McGill Cohort Study

The Ludwig-McGill Cohort Study is a large longitudinal investigation of the natural history of HPV infection and cervical neoplasia which was carried out at Ludwig Institute for Cancer Research, Sao Paulo Branch, Brazil, in collaboration with McGill University, Montreal, Canada. Its design and methods have been described in detail in previous work (165). The objectives of this prospective cohort study were : (1) study the epidemiology of persistent cervical HPV infection in asymptomatic women, (2) investigate whether persistent HPV infection increases cervical precancerous lesions, (3) search for determinants of persistent HPV infection, (4) search for molecular variants of HPV that may be associated with an increased risk of lesions, (5) investigate whether viral load is correlated with persistent infections and with lesion risk, (6) study the antibody response to HPV as a predictor of persistence and lesion progression, and (7) evaluate the involvement of patients' genetics in mediating HPV persistence and lesion severity. Study participants are described below.

2.3. Methods

2.3.1. Study participants

Out of 3,589 women eligible to be enrolled in the Ludwig-McGill cohort study, 2,528 accepted to participate in the study which resulted in a response rate of over 70%. After further review restricted to eligibility, the cohort included 2,462 participants. The study population of this work is summarized in Figure I, page 53. They belong to a subset of Brazilian women attending a comprehensive maternal and child health program catering to low-income families in the city of Sao Paulo, Brazil, from 1993 to 1997. Participants were followed up on average for 6 years with some women who were followed for up to 10 years at scheduled returns every 4 months in the first year and once every 6 months thereafter.

In brief, two nurses were employed and trained specifically for the study. They recruited participants randomly from the daily lists of outpatients in the family medicine, gynecology, and family planning clinics at the *Municipal Hospital Maternidade Escola Dr. Mario de Moraes Altenfelder Silva*, popularly known as *Maternidade Escola Vila Nova Cachoeirinha*, Sao Paulo, Brazil. The inclusion criteria were: (1) being 18–60 years old, (2) being permanent residents of Sao Paulo, (3) had no intention to become pregnant over the next year, (4) had an intact uterus without referral for hysterectomy, (5) had no treatment for cervical disease within 6 months before enrolment, and (6) reported no use of vaginal medication in the 2 days prior to enrolment. Eligible participants answered baseline and follow-up questionnaires administered by the nurses to collect information on sociodemographic, lifestyle, and sexual, reproductive, and contraceptive characteristics. Questionnaires varied between visits, with the questionnaire at baseline being the most

detailed. The codebook of the baseline questionnaire is in the appendix III. Patient's biological samples were collected at baseline and each scheduled visit. Cervical cell specimens were collected for Pap cytology and HPV DNA analyses and blood samples for HPV serology. Cervicographies were performed once within the first year for each participant at one of the first four visits as well as at 24 and 48 months.

Women recruited for the study were compensated with meal tickets which had a cash value honored by almost all shopping facilities, including groceries. To encourage compliance with follow-up visits, the value of the first meal ticket started at 5$ and increased by 5$ at each subsequent visit to a maximum of 20$ for every visit afterward. Meal ticket values were converted in US dollars to facilitate understanding.

2.3.2. Cervical specimens

An Accelon biosampler (Medscand Inc., Hollywood, FL, USA) was used to collect a sample of ecto- and endocervical cells. After preparation of the pap smear on a glass slide for cytology, remaining exfoliated cells were preserved in Tris-EDTA buffer (pH 7.4) at most 5 days at 4ºC and were then frozen. Samples were sent to the Ludwig Institute for Cancer Research in Sao Paulo for storage and testing. Pap smears were shipped to Montreal, where they were re-read by one of the Canadian collaborators. Cytopathology reports were based on the Bethesda system for cytologic diagnoses (166).

2.3.3. HPV detection and genotyping

Standard techniques were used to extract and purify DNA from cervical cells. In brief, samples were digested with 100µg/ml proteinase K for 3-18h at 55ºC, and the DNA purified by spin-column chromatography. Specimens were tested for the presence of HPV DNA by a

previously described PCR protocol amplifying a highly conserved 450 base pairs (bp) segment of the L1 viral gene flanked by MY09/11 or PGMY09/11 primers (167, 168). Genotyping of the amplified products was performed by hybridization with individual oligonucleotide probes labelled with P^{32} and specific for 27 HPV genital types whose nucleotide sequences for probes within the MY09/11 fragment have been published elsewhere (169). To verify the specificity of the hybridizations, we included more than 30 type-specific positive controls in all membranes.

Amplified products hybridizing to the generic probe, but not to any of the type-specific probes were further tested by restriction fragment length polymorphism analysis of the L1 fragment extending the range of identifiable HPV to more than 40 genital types (42). The informative enzymes for this analysis include *BamHI*, *DdeI*, *HaeIII*, *HinfI*, *PstI*, *RsaI*, and *Sau3aI*. (170). The genotypes tested included high oncogenic risk (HR-) HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82, and low oncogenic risk (LR-) HPV types 6, 11, 26, 32, 34, 40, 42, 44, 53, 54, 57, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, 89, and CP6108, plus other unknown types (20, 171). Testing for host DNA was performed using GH20 and PCO4 primers, which amplify a 268 bp region of human β-globin gene. Specimens were tested blindly with respect to all other participant-specific information and care was taken to avoid contamination in all procedures. Only samples that tested positive at least for β-globin were considered adequate and included in the analysis.

2.3.4. HPV serology

Serum samples were separated from clotted blood specimens and stored at −20°C until testing. The level of IgG antibodies to HPV16 was measured by a semi-quantitative method,

the enzyme-linked immunosorbent assay (ELISA). Recombinant HPV16 VLPs, composed by L1 only and L1 along with L2, were prepared in baculovirus (45). They were kindly provided by Dr. I. Frazer, University of Queensland, Australia and Dr. J. Schiller, National Institute of Health, United States, respectively. The ELISA protocol was performed as previously described (30, 46). Briefly, polystyrene microtiter plates were coated with 50 μL of a solution containing 2 mg of HPV16 VLP per 100 mL of PBS (phosphate-buffered saline) and incubated for 1.5 hours at 37°C. Plates were washed three times with calcium- and magnesium-free PBS and were then incubated with serum samples diluted 1:10 or 1:50 in PBS containing 0.5% skim milk and 0.1% newborn calf serum (PBS-MNCS) for 2.5 hours at 37°C. Following repeated washings, plates were incubated with 50 μL of a previously standardized dilution of peroxidase-labeled anti-IgG conjugate for 1 hour at room temperature. Following an additional washing cycle, a chromogen substrate mixture (0.1 mg/mL O-phenylenediamine and 0.003% hydrogen peroxide diluted in 0.15 mol/L PBS; pH 6.0) was added to the wells. Absorbances were read at 490 nm in a colorimetric plate reader after 45 minutes. Replicate blank wells with PBS-MNCS instead of diluted serum samples and a control human serum pool were included in all plates. The latter was included to control the inter- and intra-assay variation in reactivity that is inherent to immunoenzymatic techniques. A single batch of this serum pool was prepared in advance and used throughout the study. It was prepared from dozens of blood banks and normal clinical laboratory specimens from female adult donors at the AC Camargo Hospital in Sao Paulo. Specimens were then aliquoted and kept frozen at -20°C. Absorbances were corrected for the fluctuation in seroreactivity of the serum pool as previously described (46). Seroreactivity was expressed as normalized absorbance ratio (NAR) by dividing the mean blank-subtracted optical density (OD) by the equivalent value of

the control serum pool included in the same plate in triplicate (46). Sample size analyzed for HPV16 IgG antibodies seropositivity in this study is described in detail in the item 2.3.6, entitled statistical analysis and illustrated in the Figure I, page 53.

2.3.5. Viral load

Cervical specimens found to be positive with the main PCR protocol (MY09/11) were retested by a quantitative PCR to measure viral burden known as low-stringency PCR (LS-PCR) (172). Briefly, a consensus primer pair (GP5/GP6) targeting the L1 gene of a broad spectrum of HPV was employed under low-stringency conditions to coamplify the specific HPV DNA fragment (140 bp) along with DNA sequences from the human genome present in the starting PCR mixture (173). A 192 bp DNA product homologous to a small region of the human chromosome X was selected to serve as internal control for the reaction. DNA extracted from two cervical carcinoma cell lines with known quantities of HPV copies (HeLa, 20–40 copies/cell of HPV18 and Caski, 400–600 copies/cell of HPV16) were used as viral load controls (174). Standards were prepared with a reference HPV16 plasmid kindly provided by Dr. E.M. de Villiers, *Deutsches Krebsforschungszentrum*, Heidelberg, Germany. They consisted of mixtures containing varying amounts of the reference HPV16 plasmid (corresponding to 0, 4, 20, 100, 500, and 2,500 viral copies/cell) added to a constant background of DNA extracted from human breast tissue which were tested in all reactions.

LS-PCR components in final volume of 20 µl were: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 0.1 units of Taq DNA polymerase (Invitrogen, Grand Island, NY, USA), 10 ng of the template DNA, 200 µM of each dNTPs (dATP, dTTP, dCTP, and dGTP), and 10 pmol of each primer GP5/GP6 (GP5: 5' TTTGTTACTGTGGTAGATAC 3 ', GP6: 5'

GAAAAATAAACTGTAAATCA 3') (173). The reaction conditions were: one cycle of 94ºC for 3 minutes, 45ºC for 1 minute, and 72ºC for 1 minute, followed by 9 cycles of 92ºC for 30 seconds, 45ºC for 1 minute, and 72ºC for 1 minute, and 29 cycles of 92ºC for 30 seconds, 40ºC for 1 minute, and 72ºC for 1 minute. Finally, a cycle with 92ºC for 30 seconds, 40ºC for 1 minute, and 72ºC for 5 minutes.

Amplified products were run in silver-stained polyacrylamide gels (8%) (175). The ratio of the (140 bp) HPV band signal density to that of the internal control band (192 bp) was then measured by densitometry and quantified (in copies per cell) by linear interpolation using a standard curve constructed with the standards. Samples and controls were tested in duplicate, while standards in triplicate. Viral load was derived from the mean values. The protocol was described in details in previous work (172).

2.3.6. Statistical analysis

Descriptive statistics including median and interquartile range (IQR), mean and standard deviation (SD), and percentage were used to describe participant's characteristics for all women included in the cohort (n=2,462), those tested individually with VLP composed by L1+L2 (n=1,975) as well as those tested with both VLP types (L1 and L1+L2) (n=246) at baseline. The subset of 246 women was selected based on their HPV DNA positivity to allow the investigation of our results in a fictitious population that contains a greater number of HPV-positive women, especially those infected with HPV16 alone or with other genotypes.

Box-and-whiskers plot was presented to describe the level of HPV16 IgG antibodies (L1 vs. L1+L2 for both serum dilutions, 1:10 and 1:50) detected at baseline among the 246 women tested with both protocols and serum dilutions. In this representation, the lower

adjacent values of HPV16 IgG antibodies were computed by subtracting 1.5-fold the IQR (25^{th} percentile – 75^{th} percentile) from the first quartile (25^{th} percentile). Upper adjacent values were computed by subtracting 1.5-fold the IQR from the third quartile (75^{th} percentile).

In order to compare the ELISA protocols based on serum dilutions (1:10 vs. 1:50) and VLP types (L1 and L1+L2 VLP), we used Pearson's correlation (r) followed by its 95% CI. Linear regressions were done to add regression lines and their 95% CI in the graphics. The coefficient of determination (R^2) and its 95% CI (computed by bootstrapping) was also estimated. Bland-Altman method was also used to quantify the magnitude of differences between serum dilutions for each ELISA protocol (L1 and L1+L2 VLP) in the subset of women tested with both VLPs at baseline (n=246). For the Bland-Altman method we constructed a scatter plot containing the mean difference between serum dilutions 1:10 (measure A) and 1:50 (measure B) and its limits of agreement which were calculated using the mean difference and the standard deviation (SD) (176, 177). This method recommends that 95% of the data points should lie within ±2*SD of the mean difference or more precisely ±1.96*SD (95% limits of agreement). Normalized absorbance ratios (NARs) were log_{10}-tranformed to ensure the assumption of normality of differences which was previously verified with a histogram. The Y axis of the scatter plot shows the difference between the two paired measurements (A – B), and the X axis represents the average of these measures [(A + B)/2]. In order to better detect the proportional difference between both measurements we included in the plots the regression lines and their 95% CI computed by bootstrapping. The magnitude of differences can be quantified by the gap between the Y axis corresponding to zero difference and the parallel line to the X axis representing the observed mean difference between both measurements.

The accuracy of the protocols to detect HPV16 IgG antibodies were assessed using receiver operating characteristic (ROC) curves with HPV16 DNA infection as gold standard. Areas under the ROC curves were estimated with their 95% CI.

We used linear regression to analyze the association between HPV16 seropositivity (log_{10}-transformed NARs) and HPV DNA infection at baseline. We built 3 models of exposure focusing on HPV DNA types 16, 31, 35, 52, 67, 33, and 58, which belong to genus alpha-papillomavirus, species 9 (48). We built a first model comparing HPV16 DNA to the reference including all other HPV type or HPV-negative cases. A second model including 3 categories comparing HPV16, other alpha 9 types highly related to HPV16 (i.e., HPV31/35) versus the reference group including any other HPV type or HPV-negative women. Finally, a third model including 4 categories was used to compare women positive for HPV16, HPV31 or 35, and other alpha 9 HPV types moderately related to HPV16 (i.e., HPV52/67/33/58) versus the reference including any other HPV type or HPV-negative women (59). Using these models, we first analyzed the subset of 246 women tested with both ELISA protocols and serum dilutions. Then, we used the third model to analyze the entire cohort (excluding fourteen women who had no information on HPV status) (n=1,961).

The association between HPV16 seropositivity and HPV16 DNA infection was also analyzed as single type infection compared to co-infection with other HPV types (multiple types). In this model, HPV exposure was categorized as follow: HPV16 single infection, HPV16 co-infection with other HPV types, and the reference category including all other women (i.e., infected with HPV other than 16 or negative). We used this model to analyze the subset of 246 women tested with both ELISA protocols and serum dilutions. Then, we analyzed the entire cohort tested for HPV16 serology with the L1+L2 VLPs and serum

dilution 1:10. (n=1,961). For all linear regression analyses we provided the regression coefficients (β) and their 95% CI. Technically, β coefficients are interpreted based on the reference group, and they can be used to determine the impact of the independent variable (HPV DNA positivity) on the dependent variable (HPV16 seroreactivity). They represent the estimated change in HPV16 seroreactivity for a unit change in HPV DNA positivity. Since HPV positivity is a categorical variable, one-unit change means moving to the adjacent category. The coefficient of determination (R^2) and its 95% CI (computed by bootstrapping) was also estimated to measure how well the regression models fitted the observed data.

Using the entire cohort tested for HPV16 serology with L1+L2 VLPs and serum dilution 1:10 (n=1,961), we investigated the impact of age as potential confounder in both models constructed to evaluate HPV exposure (i.e., phylogenetic relatedness to HPV16 and type of infection - single vs. multiple). For that, we included the variable age in our models (continuous) and we then evaluated the percentage of variation between the crude and adjusted β coefficients. We used a variation of >10% in the β coefficients as indicative of confounding. An interaction parameter was also added in our models to investigate the modifying effect of age. Statistical significance was achieved if the p-value of this parameter was <0.05.

Finally, association between HPV16 DNA viral load (copies/cell) and HPV16 seropositivity was investigated among women with HPV16 single type infection detected in the cohort at baseline (n=41) using the same ELISA protocol as above (L1+L2 VLPs, serum dilution 1:10). All analyses were performed using STATA statistical software (version 14.2).

2.3.7. Power estimation

A power analysis comparing two-sample means was done using the two-sided t-test with a significance level (α) of 0.05 using the data obtained with the ELISA protocol - L1+L2

VLP and serum dilution 1:10. The estimated power of the comparative analysis was 99.74%. Parameters used for the calculation were: total sample size = 246, number of women infected with HPV16 DNA (n=28), and any other HPV DNA result (Else, n=218), ratio (218/28) = 7.78, mean HPV16 IgG NAR in women within the category "Else" = -0.14 log units, mean HPV16 IgG NAR of HPV16 DNA positive women = 0.09 log units, and SD = 0.24 log units.

The estimated power considering the entire cohort was 99.45%. Parameters used for the calculation were: total sample size = 1,961, number of women infected with HPV16 DNA (n=60), and any other HPV DNA result (Else, n=1,901), ratio (1,901/60) = 31.68, mean HPV16 IgG NAR in women within the category "Else" = -0.07 log units, mean HPV16 IgG NAR of HPV16 DNA positive women = 0.06 log units, and SD = 0.22 log units.

2.4. Ethical considerations

Women were enrolled to participate in this study only after giving signed informed consent. All study procedures and the informed consent were approved by the institutional review boards and ethical committees of the participating institutions: McGill University, Montreal, Canada, the Ludwig Institute for Cancer Research, and the *Maternidade Escola Vila Nova Cachoeirinha* clinic, the last two from Sao Paulo, Brazil. The McGill University ethics certificate has been renewed annually. The master's student also obtained ethical permission to do this work from the CERES (*Comité d'éthique de la recherche en santé*) which is the University Council Committee at the *Université de Montréal*.

All professionals and students who contribute to the accomplishment of this study have the ethical and moral obligation to keep confidential all that they have learned, seen or heard in the exercise of their work to protect study participants against stigmatization and inequalities, and they must have the same respect and concern for each of them. We were

committed to demonstrate scientific rigor at the time of the data analysis, interpretation, and communication of our results.

2.5. Contribution to the Ludwig-McGill cohort study

I have worked with the Ludwig-McGill cohort since 1997. I received my master's degree in 1999 and PhD. in 2004, both in microbiology, working on the projects "HPV viral load in clinical specimens using low-stringency PCR" and "Viral load and physical state of human papillomavirus in cervical smears", respectively, under the supervision of Dr. Luisa Lina Villa from Sao Paulo, Brazil. Among the objectives of these projects were the standardization of real-time PCRs to detect viral load targeting three HPV16 genes (E2, E6, and L1), and a protocol to detect the HPV16 physical state in cervical cells. The validation of the protocols was done by testing thousands of DNA samples from the Ludwig-McGill cohort study. Even after finishing my degree in microbiology, I continued to collaborate with the principal investigators of the study, Dr. Eduardo Franco and Dr. Luisa Lina Villa, in publications that included the results of my projects. In the fall of 2016, I decided to add in my career the experience in epidemiology. Dr. Helen Trottier and Dr. Eduardo Franco offered me the opportunity to learn epidemiological analysis with the database of the same cohort. This dissertation is the result of this learning.

Chapter 3. Manuscript

HPV DNA INFECTION AS A PREDICTOR OF HPV16 SEROPOSITIVITY FOR IgG ANTIBODIES IN THE LUDWIG-MCGILL COHORT STUDY

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RUNNING TITLE: Association between HPV16 seropositivity and HPV DNA infection

DISCLOSURE/CONFLICT OF INTEREST

Luisa Lina Villa has been consultant of Merck, Sharp & Dohme for the Quadrivalent HPV vaccine; to BD, Qiagen and Roche Molecular Diagnostics for HPV DNA tests. Eduardo L. Franco has served as consultant to Merck and GSK, on HPV vaccine matters, to BD, Roche, and Gen-Probe, on issues related to HPV diagnostics. Helen Trottier has served as consultant to Merck and GSK. The other authors disclose no conflict of interest.

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ABSTRACT

Background: Seroconversion does not always occur following HPV infections. We compared two protocols based on two serum dilutions to measure HPV16 seroreactivity and investigated if HPV DNA positivity was a correlate of HPV16 seropositivity. We also assessed if the association was influenced by co-infection with multiple HPV types and viral load.

Methods: We used baseline data of women participating in the Ludwig-McGill cohort. ELISA assays were based on L1 and L1+L2 virus-like particles (VLP). Serum dilutions were 1:10 and 1:50. Seroreactivity was expressed as normalized absorbance ratios (NAR). HPV genotyping and viral load were evaluated by PCR-based methods. Comparisons were evaluated through Pearson's correlation (r). The accuracy of the tests was compared using receiver operating characteristic (ROC) curves with HPV16 DNA positivity as gold standard. Association between HPV16 seropositivity and HPV DNA positivity was analyzed by linear regression.

Results: Assays were highly correlated ($0.87 \leq r \leq 0.94$). The protocol with the best accuracy was with L1+L2 VLPs and serum dilution 1:10 (ROC area=0.7330, 95% CI: 0.6465 – 0.8495). Regression models showed that HPV16 seropositivity was associated with HPV16 DNA positivity only, and the association was not influenced by either co-infection or viral load.

Conclusion: HPV16 DNA infection is a correlate of HPV16 seropositivity.

KEYWORDS

Human papillomavirus, natural infection, IgG antibodies, HPV16 seropositivity, HPV DNA, viral load, coinfections, virus-like particles, enzyme-linked immunosorbent assay.

INTRODUCTION

Cervical cancer ranks as the fourth most frequent malignancy among women worldwide and the second most common cancer in women aged 15 to 44 years (1, 2). Persistent HPV infection causes virtually all cervical cancer cases (3). HPV16 is the most prevalent genotype being responsible for 50% of cases (3, 4).

Most HPV infections are transient and clear within 1-2 years by the immune system (4, 5). About 60-70% of all infected women develop measurable HPV antibodies (6). HPV16 DNA-positive women tend to be more frequently seropositive than HPV DNA-negative women (7). Several studies have found a positive association between HPV16 seropositivity and HPV DNA positivity; however, some of them did not reach statistical significance (8-10).

Serological assays are useful for measuring humoral immune response of cumulative exposure to a viral infection from multiple anatomic sites (11-13). Researchers have used virus-like particles (VLP) in serological tests in the absence of efficient methods to harvest native antigens from tissue culture (14). L1 and L2 are the major and minor capsid proteins, respectively. L1 alone or with L2 recombinantly expressed self assembles into VLPs lacking the viral genome. They are structurally similar to authentic virions (11-13). Little is known if L1+L2 VLPs performs better than L1 only and if they can be responsible for cross-reactivity between HPV types in immunoassays (14-18).

We compared two protocols based on two serum dilutions to measure total HPV16 IgG antibodies in a cohort of Brazilian women naturally infected with HPV. We also investigated if HPV DNA positivity was associated with HPV16 seropositivity in this cohort, and if the association was influenced by co-infection with other HPV types and viral load.

METHODS

Study participants

The Ludwig-McGill Cohort Study is a large longitudinal investigation of the natural history of HPV infection and cervical neoplasia. The study enrolled 2,462 Brazilian women from 1993 to 1997 (Figure I, page 53). They were women attending a comprehensive maternal and child health program catering to low-income families in the city of Sao Paulo, Brazil. The design and methods of the study have been described previously (19)**.** In brief, two nurses trained specifically for the study recruited participants by selecting them at random from the daily lists of outpatients in the family medicine, gynecology, and family planning clinics at *Maternidade Escola Vila Nova Cachoeirinha*, Sao Paulo, Brazil. The inclusion criteria were: (1) being 18–60 years old, (2) being permanent resident of Sao Paulo, (3) had no intention to become pregnant over the next year, (4) had an intact uterus without referral for hysterectomy, (5) had no treatment for cervical disease within 6 months before enrolment, and (6) reported no use of vaginal medication in the 2 days prior to enrolment. Participants were followed up to 10 years at scheduled returns every 4 months in the first year and once every 6 months thereafter. Cervical cell specimens were collected for Pap cytology and HPV DNA analyses, and blood samples for HPV serology at baseline and each scheduled visit. Eligible participants signed an informed consent and answered baseline and follow-up nurse-administered questionnaires to collect information on sociodemographic, lifestyle, and sexual, reproductive, and contraceptive characteristics. The study protocol was approved by the ethical review boards of the participating institutions in Canada and Brazil.

Cervical cell specimens

An Accelon biosampler (Medscand Inc., Hollywood, FL, USA) was used to collect a sample of ecto- and endocervical cells. After preparation of the Pap smear on a glass slide for cytology, remaining exfoliated cells were preserved in Tris-EDTA buffer (pH 7.4) at 4ºC at most 5 days and were then frozen until testing.

HPV detection and typing

Standard techniques were used to extract and purify DNA from cervical cells. In brief, samples were digested with 100µg/ml proteinase K for 3-18h at 55ºC, and the DNA purified by spin-column chromatography. Specimens were tested for the presence of HPV DNA by a previously described PCR protocol amplifying a highly conserved 450 base pairs (bp) segment of the L1 viral gene flanked by MY09/11 or PGMY09/11 primers (20, 21). Genotyping of the amplified products was performed by hybridization with individual oligonucleotide probes labelled with P^{32} and specific for 27 HPV genital types (22). Amplified products hybridizing to the generic probe, but not to any of the type-specific probes were further tested by restriction fragment length polymorphism analysis using the restriction enzymes, extending the range of identifiable HPV to more than 40 genital types (23). Testing for host DNA was performed using GH20 and PCO4 primers, which amplify a 268 bp region of human β-globin gene (24). The genotypes tested included HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82, and low oncogenic risk (LR-) HPV types 6, 11, 26, 32, 34, 40, 42, 44, 53, 54, 57, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, 89, and CP6108, plus other unknown types (25). Specimens were tested blindly with respect to all other participant-specific information and care was taken to avoid contamination in all procedures. Only samples that tested positive at least for β-globin were considered adequate and included in the analysis.

HPV serology

Serum samples were separated from clotted blood specimens and stored at -20°C until testing. The level of HPV16 IgG antibodies was measured by a semi-quantitative method, the enzyme-linked immunosorbent assay (ELISA). Recombinant HPV16 VLPs, expressing L1 only and L1 with L2, were prepared in baculovirus (26). They were kindly provided by Dr. Ian Frazer, University of Queensland, Australia, and Dr. John Schiller, National Institute of Health, United States, respectively. The ELISA protocol was performed as described in previous work (27, 28). Briefly, polystyrene microtiter plates were coated with 50 μL of a solution containing 2 mg of HPV16 VLP per 100 mL of PBS (phosphate-buffered saline) and incubated for 1.5 hours at 37°C. Plates were washed three times with calcium- and magnesium-free PBS and were then incubated with serum samples diluted 1:10 or 1:50 in PBS containing 0.5% skim milk and 0.1% newborn calf serum (PBS-MNCS) for 2.5 hours at 37°C. Following repeated washings, plates were incubated with 50 μL of a previously standardized dilution of peroxidase-labeled anti-IgG conjugate for 1 hour at room temperature. Following an additional washing cycle, a chromogen substrate mixture (0.1 mg/mL O-phenylenediamine and 0.003% hydrogen peroxide diluted in 0.15 mol/L PBS; pH 6.0) was added to the wells. Absorbances were read at 490 nm in a colorimetric plate reader after 45 minutes. Replicate blank wells with PBS-MNCS instead of diluted serum samples and a control human serum pool were included in all plates. The latter was included to control the inter- and intra-assay variation in reactivity that is inherent to immunoenzymatic techniques. A single batch of this serum pool was prepared in advance and used throughout the study. It was prepared from dozens of blood banks and normal clinical laboratory specimens from female adult donors at the AC Camargo Hospital in Sao Paulo. Specimens were then aliquoted and kept frozen at -20°C. Absorbances were

corrected for the fluctuation in seroreactivity of the serum pool as previously described (28). Seroreactivity was expressed as normalized absorbance ratio (NAR) by dividing the mean blank-subtracted optical densities (OD) by the equivalent value of the control serum pool included in the same plate in triplicate. This method is used to minimize measurement error in ELISA assays (28). Sample size analyzed for IgG antibodies seropositivity in this study is described in Figure I, page 53.

Viral load

Cervical specimens found to be positive with the main PCR protocol (MY09/11) were retested by a quantitative PCR to measure viral burden (29). Briefly, a consensus primer pair (GP5, GP6) targeting the L1 gene of a broad spectrum of HPV was employed under low-stringency conditions to coamplify the specific HPV DNA fragment (140 bp) along with DNA sequences from the human genome present in the starting PCR mixture (30). A 192 bp DNA product homologous to a small region of the human chromosome X was selected to serve as internal control for the reaction. DNA extracted from two cervical carcinoma cell lines with known quantities of HPV copies (HeLa, 20–40 copies/cell of HPV18 and Caski, 400–600 copies/cell of HPV16) were used as viral load controls (31). Standards consisting of mixtures containing varying amounts of a reference HPV16 plasmid (corresponding to 0, 4, 20, 100, 500, and 2,500 viral copies/cell) added to a constant background of DNA extracted from human breast tissue were tested in all reactions. Viral load was quantified by linear interpolation using the standard curve. Samples and controls were tested in duplicate, while standards in triplicate, viral load (in copies per cell) was derived from the mean values. The protocol was described in details in previous work (29).

Statistical analysis

Descriptive statistics including median and interquartile range (IQR), mean and standard deviation (SD), and percentage were used to describe participant's characteristics for all women included in the cohort (n=2,462), those tested individually with VLP composed by L1+L2 (n=1,975) as well as those tested with both VLP types (L1 and L1+L2) (n=246) at baseline. The subset of 246 women was selected based on their HPV DNA positivity to allow the investigation of our results in a fictitious population that contains a greater number of HPV-positive women, especially those infected with HPV16 alone or with other genotypes.

Box-and-whiskers plot was presented to describe the level of HPV16 IgG antibodies detected at baseline among the 246 women tested for both protocols and serum dilutions. In this representation, the lower adjacent values of HPV16 IgG antibodies were computed by subtracting 1.5-fold the IQR (25^{th} percentile – 75^{th} percentile) from the first quartile (25^{th} percentile). Upper adjacent values were computed by subtracting 1.5-fold the IQR from the third quartile (75^{th} percentile). In order to compare the ELISA protocols based on serum dilutions (1:10 vs. 1:50) and VLP types (L1 and L1+L2 VLP), we used Pearson's correlation (r) followed by its 95% CI. Linear regressions were done to add regression lines and their 95% CI in the graphics. The coefficient of determination (R^2) and its 95% CI (computed by bootstrapping) was also estimated.

The accuracy of the protocols to detect HPV16 IgG antibodies were assessed using receiver operating characteristic (ROC) curves using HPV16 DNA infection as gold standard. We used linear regression to analyze the association between HPV16 seropositivity (log_{10}-transformed NARs) and HPV DNA positivity at baseline. For all linear regression analyses we provided the regression coefficients (β) and their 95% CI. β coefficients represent the estimated change in HPV16 seroreactivity for a unit change in HPV DNA positivity. The coefficient of

determination (R^2) and its 95% CI (computed by bootstrapping) was also estimated to measure how well the regression models fitted the observed data.

We built 3 models of exposure focusing on HPV DNA types 16, 31, 35, 52, 67, 33, and 58, which belong to genus alpha-papillomavirus, species 9 (32). We built a first model comparing HPV16 DNA to the reference including all other HPV type or HPV-negative cases. A second model including 3 categories comparing HPV16, other alpha 9 types highly related to HPV16 (i.e., HPV31/35) versus the reference group including any other HPV type or HPV-negative women. Finally, a third model including 4 categories was used to compare women positive for HPV16, HPV31 or 35, and other alpha 9 HPV types moderately related to HPV16 (i.e., HPV52/67/33/58) versus the reference including any other HPV type or HPV-negative women (33). Using these models, we first analyzed the subset of 246 women tested with both ELISA protocols and serum dilutions. Then, we used the third model to analyze the entire cohort (n=1,961).

Finally, we analyzed the relationship between HPV16 serology and HPV DNA positivity using linear regression in the entire cohort (n=1,961) tested with L1+L2 VLPs and serum dilution 1:10 (excluding fourteen women who had no information on HPV status). We analyzed HPV16 DNA infection as single type infection compared to co-infection with other HPV types (multiple types). HPV exposure was categorized as follow: HPV16 single infection, HPV16 co-infection with other HPV types, and the reference category including all other women (i.e., infected with HPV other than 16 or negative). Association between HPV16 DNA viral load (copies/cell) and HPV16 seropositivity was also investigated using Pearson's correlation among women with HPV16 single type infection detected in the cohort at baseline (n=41) using the same ELISA protocol as above (L1+L2 VLPs, serum dilution 1:10). The

impact of age as potential confounder or effect modifier was analyzed in both models used to evaluate HPV exposure (i.e., phylogenetic relatedness to HPV16 and type of infection - single vs. multiple). Analyses were performed using STATA statistical software (version 14.2).

RESULTS

The Ludwig-McGill cohort included 2,462 participants. The mean follow-up time (SD, years) was 6.37 (±1.99), median (IQR) 7.09 (6.20 – 7.50). HPV16 seropositivity using VLPs composed by L1+L2 was tested in 1,975 women at baseline. Out of them, 246 were tested for the level of HPV16 IgG antibodies by the two ELISA protocols differing from each other by the composition of the VLP used as antigens (L1 or L1+L2) and based on two serum dilutions (1:10 and 1:50) (Figure I, page 53).

The subset of 1,975 women tested for HPV16 seropositivity was quite representative of the entire cohort, while the subset of 246 women was inflated with respect to HPV infections (HPV status, type of infection, and number of HPV types detected per women). Characteristics of all participants under investigation are described in Table 1, page 54. Normalized absorbance ratios (NARs), which represent the level of IgG antibodies produced after HPV16 natural infection, are very low independently of the protocol used (median ranged from 0.77 to 1.18) (Figure II, page 55). We observed a difference between protocols in the ability to detect higher absolute values of NARs (see upper adjacent values), while this observation is less pronounced in lower values (see lower adjacent values). HPV16 IgG NARs obtained through VLPs with L1 only were slightly higher than those obtained with L1+L2. The protocol using L1 VLPs and serum dilution 1:50 reached higher levels of HPV16 IgG antibodies compared to the protocol with L1+L2 VLPs (both serum dilutions). However, it tended to produce more outliers than the serum dilution 1:10. We observed strong correlations between results obtained

by serum dilutions 1:10 and 1:50 using both VLP types. The β coefficient of both regression lines was very similar (0.65 vs. 0.74) (Figure III A and B, page 56). On the other hand, we observed poor correlations between VLP types using the same serum dilution. Very similar β coefficient of both regression lines were observed (0.56 vs. 0.46) (Figure III C and D, page 56).

Figure IV on page 57 shows the receiver operating characteristic (ROC) curves. The best area under ROC curve was reached by the protocol using L1+L2 VLPs and serum dilution 1:10 (ROC area=0.7330, 95% CI: 0.6465 – 0.8495), although differences between ROC areas were not statistically significant as indicated by the overlapping confidence intervals. Both protocols behaved similarly when very low levels of HPV16 antibodies were detected.

Based on model 3 (both serum dilutions) used to analyze the subset of 246 women, we observed that the β coefficients of the group containing HPV types highly (HPV31/35) related to HPV16 were very close to the zero value and were not statistically significant to confirm their association with HPV16 IgG antibodies. The β coefficients of the group containing HPV types moderately (HPV52/67/33/58) related to HPV16 were higher, but still not statistically significant (Table 2, page 58). Only HPV16 DNA was associated with HPV16 IgG antibodies (with both protocols and serum dilutions) in our analysis. However, L1+L2 VLPs were better to capture the association between HPV16 seropositivity and HPV16 DNA positivity in our samples compared to VLPs composed by L1. After transforming the HPV16 NARs in $\log_{10}$ units to ensure normality of our data, we observed that HPV16 positive women were more susceptible to seroconversion compared to the reference group (seroreactivity was measured by VLP L1+L2, serum dilution 1:10). Technically, HPV16 positive women had 0.24 $\log_{10}$ units of NAR (antibody levels) higher than the reference group (Table 2, page 58). In the subset of 246 women, co-infection with multiple HPV types significantly decreased susceptibility to

seroconversion according to the test with best accuracy (L1+L2 VLP, serum dilution 1:10): β (HPV16 single infection) = 0.27 (95% CI: 0.15 – 0.40), and β (multiple HPV infection with HPV16) = 0.17 (95% CI: 0.02 – 0.32). Results obtained using linear regression were similar when we used the entire cohort tested for HPV16 seroreactivity using L1+L2 VLP and sera diluted 1:10 (n=1,961, considering missing data on HPV status) (Table 3, page 59). HPV16 DNA positive women had 0.14 $\log_{10}$ units of NAR higher than the reference group. Positivity for HPV types 31 and 35 did not significantly change the susceptibility of seroconversion, but positivity for HPV52, 67, 33 or 58 slightly did it compared to the reference group. The susceptibility of seroconversion of women co-infected with multiple HPV types was very similar to those infected with HPV16 only (Table 3, page 59). Adjustment for age did not change considerably the strength of the association observed in crude analyses. Age was not an effect modifier as the introduction of an interaction term for age in our models was not statistically significant ($p > 0.05$) (data not shown).

Finally, HPV16 viral load was not associated with HPV16 IgG antibodies. Median (IQR) of HPV16 viral load (copies/cell) was 2.0 (0.5 – 77.0), whereas the mean (SD) was 154.0 (428.9) with a minimum value of 0.5, and a maximum value of 1,940. There was no association between HPV16 viral load and seropositivity [r = -0.04 (95% CI: -0.34 – 0.27); β coefficient = -0.01 (95% CI: -0.08 – 0.06), R^2=0.02 (95% CI: -0.05 – 0.06)].

DISCUSSION

Although comparisons between serological assays have been done to measure antibody responses after HPV vaccination, studies evaluating the influence of the VLPs composition used as antigen in ELISA protocols to measure humoral immune response against naturally acquired HPV infection are lacking in the literature (14-17). The Ludwig-McGill cohort study

provides a unique opportunity to evaluate the association between HPV16 naturally acquired immunity and DNA infection in a large sample size of women collected in the pre-vaccine era.

Our comparative analysis between different ELISA protocols showed, as expected, that both methods and both serum dilutions detected very low levels of HPV16 IgG antibodies following natural HPV infection compared to the level that could be detected after vaccination (6, 34). This weak natural immune response is probably related to the absence of viremia (35).

The L1 gene has the most conserved nucleotide sequence of the HPV genome. It can be aligned for all known papillomaviruses (33). Although L2 is not very immunogenic, antibodies against L1+L2 VLPs may block infection of a diverse range of other HPV genotypes in contrast to VLPs L1 only (36). Technically, there is an increase in the yield of HPV16 VLPs when they are produced with L1 and L2 compared to L1 only which is an advantage for researchers planning to produce VLPs for their own serologic assays (37). Therefore, we investigated if the structure of the VLP used in the ELISA assays could affect the detection of HPV16 IgG antibodies. Our findings are supported by another study that compared Luminex multiplex assays performed with both VLP types. They showed that L1+L2 VLPs performed better at measuring HPV16 and 18 antibodies in large samples (14). Our results also showed a strong correlation between data obtained by serum dilutions 1:10 and 1:50 using both VLP types. Although results obtained with L1+L2 VLPs were more scattered around the regression line, they were also more stable between serum dilutions compared to L1 VLPs. In addition, we have found that results obtained from L1 and L1+L2 VLPs cannot be pooled in the same analysis since the correlation between them is poor, independently of the serum dilution used.

In general, sensitivity of ELISA protocols using VLPs as antigens is between 50 to 60% with high specificity (>90%) and good agreement between interlaboratory tests (38). This

variation in sensitivity may be due to different definitions of cut-off values between studies making the comparison between them even more difficult (35). The strength of the association between HPV16 antibodies and naturally acquired HPV DNA infection has been mostly investigated considering seropositivity as a predictor of HPV infection using logistic regression or generalized estimating equation (8-10, 27, 39-42). We evaluated HPV infection as a predictor of seropositivity through linear regression in order to avoid using a cut-off for NARs. Residuals were randomly distributed which supported the application of this model in our analysis. Both linear and logistic regressions analyses show the association between HPV16 seropositivity and HPV16 DNA positivity in cross-sectional studies (7).

HPV16 DNA positivity was considered as an independent determinant of HPV16 seropositivity in our study which is similar to the findings of others (9, 10, 27, 41-43). We have observed a low degree of cross-reactivity for infections with other alpha 9 HPV types; but the β coefficients were not statistically significant. Our results agree with others (7). Although it is not clear in the literature what are the potential confounders of this association, we analyzed the impact of age as a potential confounder and effect modifier using the entire cohort (n=1,961) (13, 27, 39, 41, 42). Age was neither a strong confounder nor an effect modifier.

It is also very common to find co-infections with multiple HPV types in many epidemiological studies (9, 27, 44). In the Ludwig-McGill cohort 12.3% of all study participants were tested positive for multiple HPV types at baseline (45). The association measured for HPV16 with multiple types (co-infection) was similar to that for HPV16 single infection. It is possible that co-infections with multiple HPV types or high HPV16 viral load reflect the inability of the immune system to respond to the viral infection (leading to low levels of antibodies), as others observed through logistic regression (9, 27). In our models,

neither HPV16 viral load nor co-infections with other types seemed to influence the association between HPV16 seropositivity and HPV16 DNA positivity.

In conclusion, our findings show that there is a positive association between HPV16 seropositivity and HPV16 DNA positivity that seems not be affected by co-infections or viral load. HPV types related to HPV16, such as HPV31, 35, 52, 67, 33 or 58 seem to not be associated with HPV16 IgG antibodies. The protocol using L1+L2 VLPs and serum dilution 1:10 better capture the association between HPV16 seropositivity and HPV16 DNA positivity. Finally, further studies are needed to investigate the association between HPV16 natural acquired immunity and co-infections, development of precursor cervical lesions, reinfection, and viral load over time.

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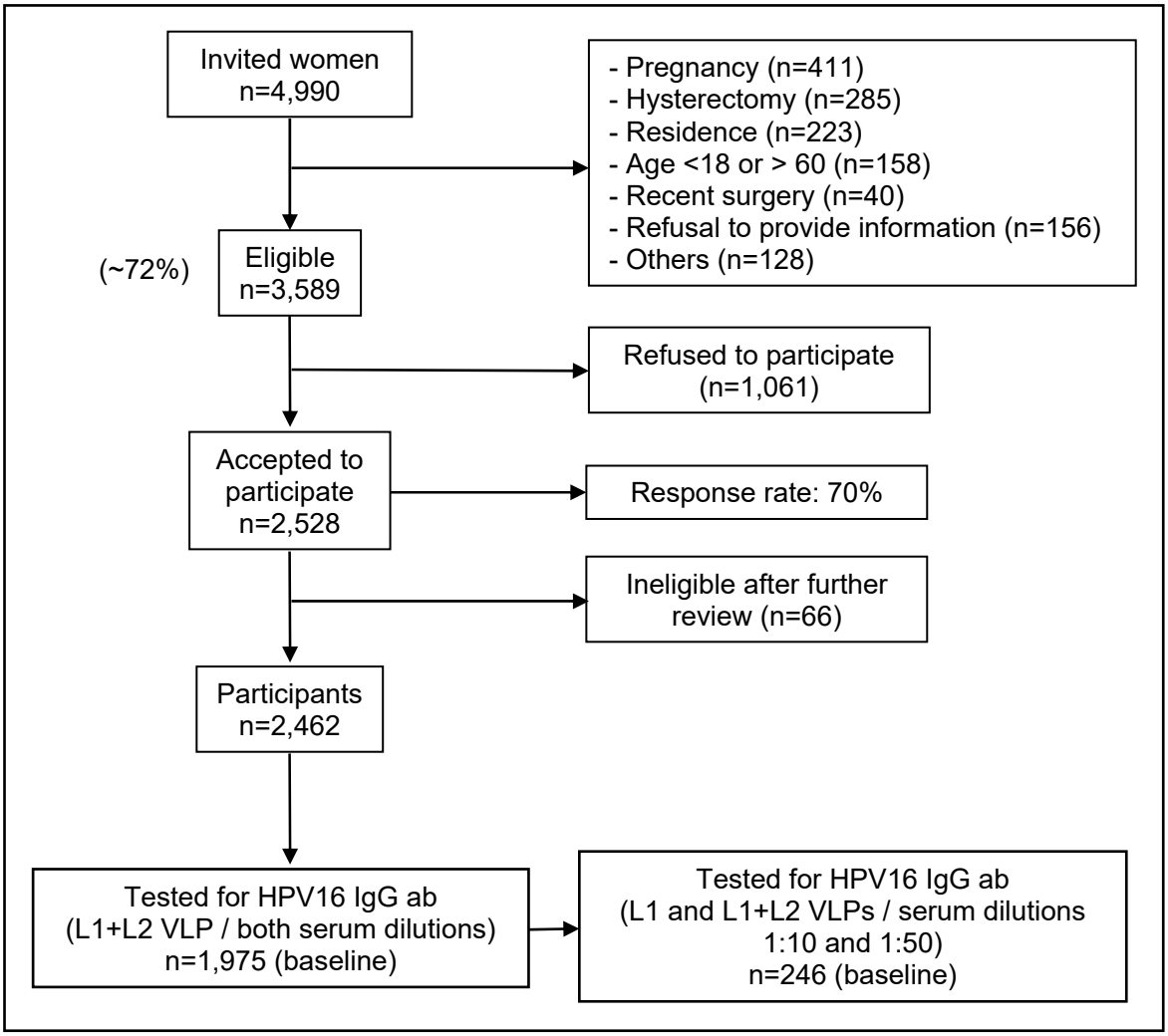
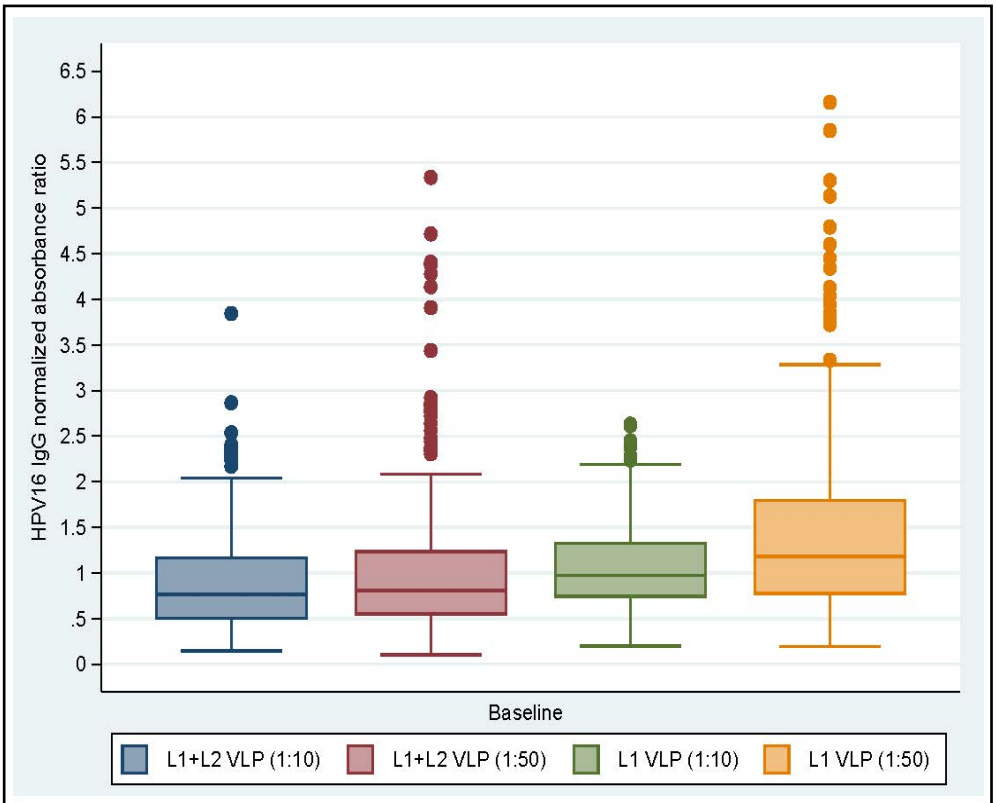


Figure I: Flowchart of the Ludwig-McGill cohort study participants. Inspired by Shaw et al., 2016 (46).

Table 1: Characteristics of the Ludwig-McGill cohort participants at baseline.

Characteristics	All participants (n=2,462)	Groups tested for HPV16 IgG	
		With L1+L2 VLP (n=1,975)	With L1+L2 and L1 VLP (n=246)
Age, yr			
Mean (SD)	32.7 (8.8)	32.9 (8.7)	33.0 (8.6)
Median (IQR)	32.0 (26.0-39.0)	32.0 (26.0-39.0)	32.0 (27.0-39.0)
Ethnicity, n (%)			
White	1,585 (64.4)	1,280 (64.8)	162 (65.9)
Others	874 (35.5)	694 (35.1)	84(34.1)
Marital status, n (%)			
Single	252 (10.2)	201 (10.2)	29 (11.8)
Cohabiting	832 (33.8)	642 (32.5)	85 (34.5)
Married	1,179 (47.9)	969 (49.1)	106 (43.1)
Separated	140 (5.7)	121 (6.1)	15 (6.1)
Widowed	57 (2.3)	42 (2.1)	11 (4.5)
Education, n (%)			
< Elementary	554 (22.5)	442 (22.4)	57 (23.2)
Elementary	1,438 (58.4)	1,164 (58.9)	147 (59.8)
Secondary	397 (16.1)	310 (15.7)	34 (13.8)
Higher education	70 (2.9)	57 (2.9)	7 (2.8)
Smoking, n (%)			
No	1,168 (47.4)	953 (48.3)	114 (46.3)
Smoker	864 (35.1)	674 (34.1)	91 (37.0)
Former	429 (17.4)	348 (17.6)	41 (16.7)
Alcohol consumption, n (%)			
No	852 (34.6)	664 (33.6)	75 (30.5)
Yes	1,601 (65.0)	1,306 (66.1)	171 (69.5)
Age at first sexual intercourse, yr			
Mean (SD)	17.9 (4.0)	17.9 (4.0)	17.9 (4.6)
Median (IQR)	17.0 (15.0-20.0)	17.0 (15.0-20.0)	17.0 (15.0-20.0)
Lifetime number of sexual partners, n (%)			
0-1	1,089 (44.2)	870 (44.0)	106 (43.1)
2-3	856 (34.8)	691 (35.0)	93 (37.8)
≥ 4	515 (20.9)	413 (20.9)	47 (19.1)
HPV status, n (%)			
Negative	2,026 (82.3)	1,629 (82.5)	183 (74.4)
Low-risk types	156 (6.3)	117 (5.9)	17 (6.9)
HPV16	67 (2.7)	60 (3.0)	28 (11.4)
HPV31 or 35	37 (1.5)	31 (1.6)	5 (2.0)
HPV52, 67, 33 or 58	46 (1.9)	40 (2.0)	7 (2.9)
Other high-risk types	107 (4.3)	84 (4.3)	6 (2.4)
Type of infection, n (%)			
Else	2,373 (96.4)	1,901 (96.9)	218 (88.6)
HPV16 single infection	45 (1.8)	41 (2.1)	17 (6.9)
Multiple HPV infection with HPV16	22 (1.0)	19 (1.0)	11 (4.5)
Number of HPV types per women, n (%)			
0	2,048 (83.2)	1,642 (83.1)	183 (74.4)
1	336 (13.6)	269 (13.6)	46 (18.7)
2	63 (2.5)	52 (2.6)	11 (4.5)
≥ 3	14 (0.6)	11 (0.6)	6 (2.4)
HPV16 viral load, copies/cell			
n (mean, SD)	66 (436.4, 1,993.4)	59 (476.8, 2,105.3)	27 (324.7, 911.8)
n (median, IQR)	66 (5.0, 0.5-86.0)	59 (5.0, 0.5-89.0)	27 (7.0, 0.5-160.0)

The number of missing values represents less than 1%. yr: years; SD: Standard deviation; IQR: Interquartile range.



	L1+L2 VLP (1:10)	L1+L2 VLP (1:50)	L1 VLP (1:10)	L1 VLP (1:50)
Median	0.77	0.81	0.97	1.18
Interquartile range	0.51 – 1.16	0.55 – 1.24	0.74 – 1.33	0.77 – 1.79
Lower adjacent value	0.14	0.10	0.20	0.19
Upper adjacent value	2.04	2.08	2.19	3.28
Minimum	0.14	0.10	0.20	0.19
Maximum	3.84	5.34	2.63	6.16

Figure II: Box-and-whiskers representation of untransformed HPV16 IgG normalized absorbance ratios (NAR) at baseline. Protocols differ by the composition of the virus-like particles (L1 and L1+L2) and are based on two serum dilutions (1:10 and 1:50) (n=246). Boxes extend from the 25th percentile to the 75th percentile (i.e., the interquartile range, IQR); lines inside boxes represent median values. Lines emerging from boxes (i.e., the whiskers) extend to the upper and lower adjacent values which are the lower and upper limits of the array, respectively. Values outside these limits are outliers represented by symbols (circle, diamond, square and triangle).

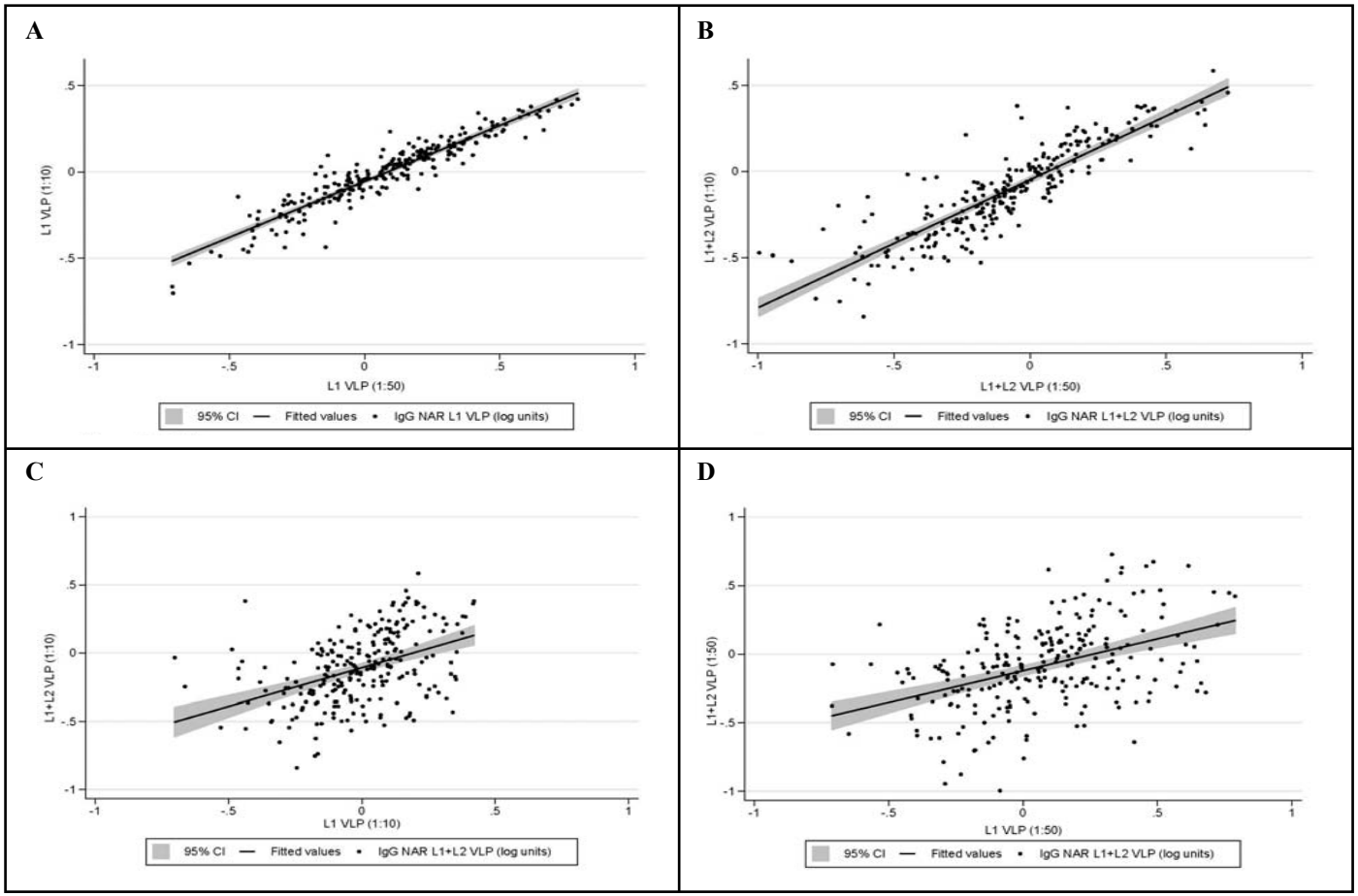
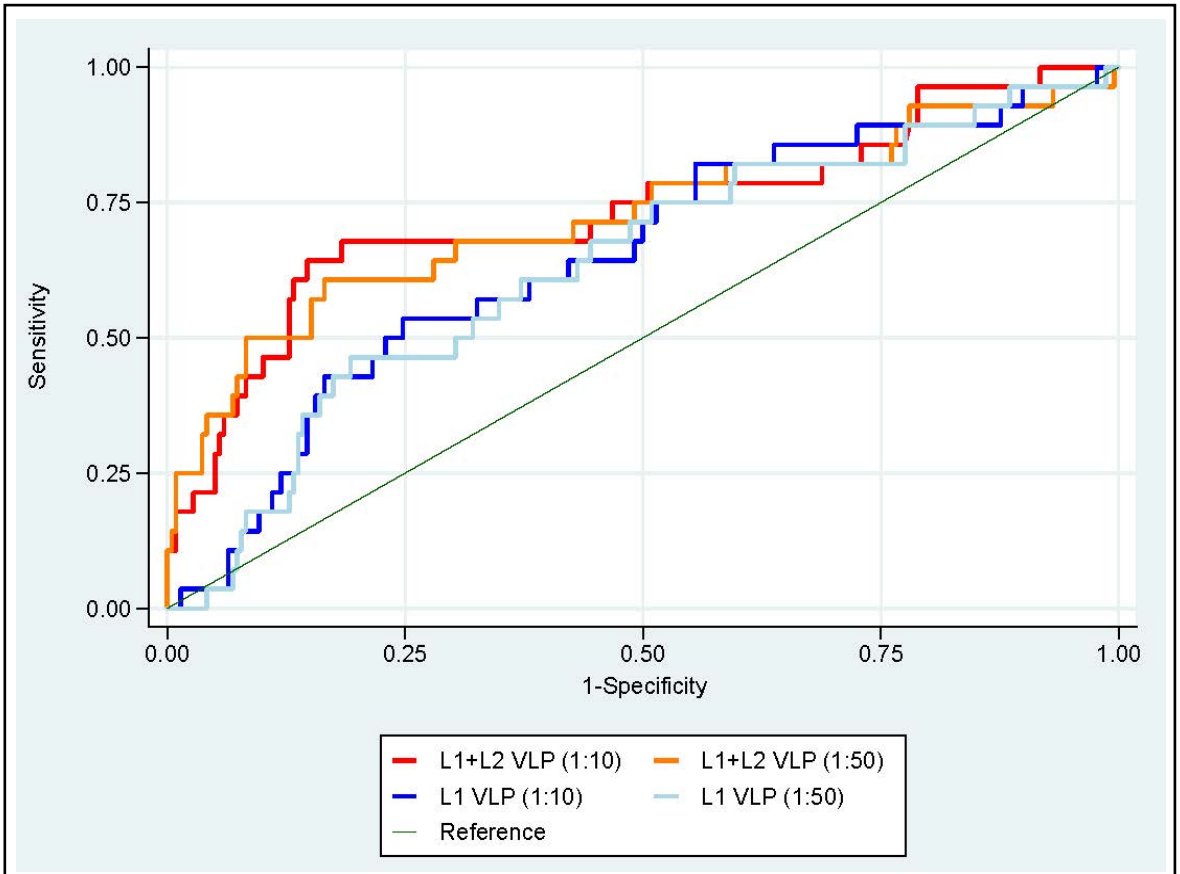


Figure III: Person's correlation (r) between log_{10}-transformed IgG normalized absorbance ratios (NAR) by serum dilution and by VLP type at baseline. Analyzed in women tested with both virus-like particles, n=246. **A.** L1 Virus-like particle (VLP), serum dilution 1:10 vs 1:50: r (95% CI) = 0.94 (0.92 – 0.95), β (95% CI) = 0.65 (0.62 – 0.67), R^2 (95% CI) = 0.88 (0.84 – 0.91). **B.** L1+L2 VLP, serum dulution 1:10 vs 1:50: r (95% CI) = 0.87 (0.84 – 0.90), β (95% CI) = 0.74 (0.69 – 0.79), R^2 (95% CI) = 0.76 (0.69 – 0.82). **C.** L1+L2 VLP vs L1 VLP, serum dilution 1:10: r (95% CI) = 0.43 (0.33 – 0.53), β (95% CI) = 0.56 (0.41 – 0.71), R^2 (95% CI) = 0.19 (0.09 – 0.28). **D.** L1+L2 VLP vs L1 VLP, serum dilution 1:50: r (95% CI) = 0.44 (0.33 – 0.54), β (95% CI) = 0.46 (0.34 – 0.58), R^2 (95% CI) = 0.19 (0.11 – 0.28).



Protocol	ROC area	95% CI
L1+L2 VLP (1:10)	0.7330	0.6465 – 0.8495
L1+L2 VLP (1:50)	0.7231	0.6016 – 0.8445
L1 VLP (1:10)	0.6483	0.5480 – 0.7584
L1 VLP (1:50)	0.6348	0.5229 – 0.7467

Figure IV: Receiver operating characteristic (ROC) curves of untransformed IgG normalized absorbance ratios (NAR). ROC areas and their respective 95% CI are presented. Protocols differ by the structure of the virus-like particles (L1 and L1+L2) and are based on two serum dilutions (1:10 and 1:50) (n=246).

Table 2: Linear regression between HPV16 seroreactivity and HPV status based on the phylogenetic relatedness to HPV16 at baseline evaluated by three models of exposure.

			L1 VLP (1 :10)	L1 VLP (1 :50)	L1+L2 VLP (1 :10)	L1+L2 VLP (1 :50)
Model	**Parameters**	**n (%)**	**β coefficient (95% CI)**	**β coefficient (95% CI)**	**β coefficient (95% CI)**	**β coefficient (95% CI)**
1	**Constant (β_0)**		-0.02 (-0.04 – -0.01)	0.06 (0.02 – 0.10)	-0.14 (-0.17 – -0.11)	-0.12 (-0.16 – -0.08)
	Else	218 (88.6)	Reference	Reference	Reference	Reference
	HPV16	28 (11.4)	0.09 (0.01 – 0.17)	0.12 (0.00 – 0.23)	0.23 (0.14 – 0.33)	0.26 (0.15 – 0.38)
	R^2 (95% CI)		0.02 (-0.02 – 0.06)	0.02 (-0.02 – 0.05)	0.08 (0.01 – 0.16)	0.08 (-0.01 – 0.16)
2	**Constant (β_0)**		-0.02 (-0.05 – 0.01)	0.06 (0.02 – 0.10)	-0.14 (-0.17 – -0.11)	-0.12 (-0.16 – -0.10)
	Else	213 (86.6)	Reference	Reference	Reference	Reference
	HPV16	28 (11.4)	0.09 (0.01 – 0.17)	0.12 (0.00 – 0.23)	0.23 (0.14 – 0.33)	0.27 (0.15 – 0.38)
	HPV31/35	5 (2.0)	0.02 (-0.16 – 0.20)	-0.05 (-0.30 – 0.20)	0.02 (-0.20 – 0.24)	0.07 (-0.18 – 0.34)
	R^2 (95% CI)		0.02 (-0.02 – 0.06)	0.02 (-0.02 – 0.05)	0.08 (0.01 – 0.16)	0.08 (-0.01 – 0.17)
3	**Constant (β_0)**		-0.02 (-0.04 – 0.01)	0.06 (0.02 – 0.10)	-0.15 (-0.18 – -0.11)	-0.12 (-0.16 – -0.08)
	Else	206 (83.7)	Reference	Reference	Reference	Reference
	HPV16	28 (11.4)	0.09 (0.01 – 0.16)	0.11 (-0.00 – 0.23)	0.24 (0.14 – 0.34)	0.27 (0.15 – 0.39)
	HPV31/35	5 (2.0)	0.02 (-0.16 – 0.19)	-0.05 (-0.30 – 0.20)	0.03 (-0.19 – 0.25)	0.08 (-0.18 – 0.34)
	HPV52/67/33/58	7 (2.9)	-0.05 (-0.20 – 0.10)	-0.08 (-0.29 – 0.14)	0.15 (-0.04 – 0.34)	0.10 (-0.12 – 0.32)
	R^2 (95% CI)		0.02 (-0.02 – 0.06)	0.02 (-0.02 – 0.06)	0.09 (0.01 – 0.17)	0.08 (-0.01 – 0.17)

Protocols differ by the structure of the virus-like particles (L1 and L1+L2) and are based on two serum dilutions (1:10 and 1:50)**. Model 1:** HPV16 positivity versus the reference including any other HPV infection with other type or HPV negative; **Model 2:** HPV16 and HPV positivity for alpha 9 types highly related to HPV16 (HPV31 or 35) versus the reference including any other HPV type with other type or HPV negative; **Model 3:** HPV16 and HPV31 or 35 and HPV positivity for alpha 9 types (moderately related to HPV16 (HPV52, 67, 33 or 58) versus the reference including any other HPV type with other type or HPV negative. IgG normalized absorbance ratios (NAR) were $\log_{10}$-transformed (n=246).

Table 3: Linear regression statistics of the HPV16 seroreactivity by HPV status at baseline in the entire cohort.

Model 1: Phylogenetic relatedness to HPV16		
Parameters	**ß Coefficients**	
	Crude (95% CI)	**Age-adjusted (95% CI)**
Constant	-0.07 (-0.08 – -0.06)	-0.16 (-0.20 – -0.12)
Else	Reference	Reference
HPV16	0.13 (0.08 – 0.19)	0.14 (0.08 – 0.20)
HPV31 or 35	0.04 (-0.03 – 0.12)	0.06 (-0.02 – 0.14)
HPV52, 67, 33, or 58	0.07 (0.00 – 0.14)	0.08 (0.01 – 0.15)
Age	-	0.00 (0.00 – 0.00)
R^2 (95% CI)	0.01 (0.00 – 0.02)	0.02 (0.01 – 0.04)
Model 2: Type of infection: single vs multiple		
Parameters	**ß Coefficients**	
	Crude (95% CI)	**Age-adjusted (95% CI)**
Constant	-0.07 (-0.08 – -0.06)	-0.15 (-0.19 – -0.11)
Else	Reference	Reference
HPV16 single infection	0.14 (0.07 – 0.21)	0.14 (0.08 – 0.21)
Multiple HPV infection with HPV16	0.11 (0.01 – 0.21)	0.12 (0.02 – 0.22)
Age	-	0.00 (0.00 – 0.00)
R^2 (95% CI)	0.01 (-0.00 – 0.02)	0.02 (0.01 – 0.03)

Log_{10}-transformed data obtained by the ELISA protocol using L1+L2 VLP and serum dilution 1:10 (n=1,961); IgG NAR: median (IQR)=0.89 (0.62 – 1.23).

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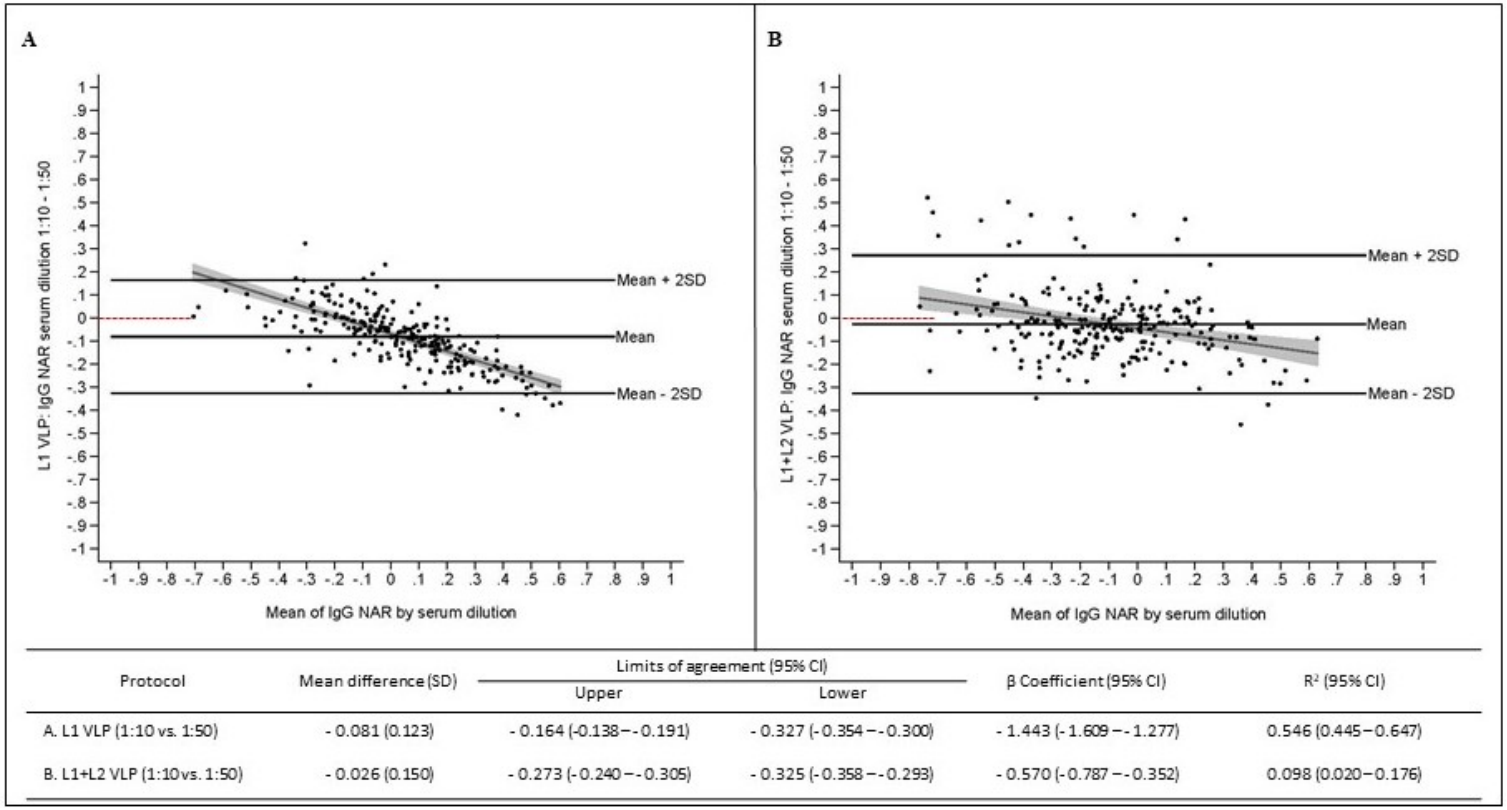
Chapter 4. Supplemental results

In this chapter are presented some supplemental results that were not included in the manuscript. The cohort tested for HPV16 IgG seroreactivity at baseline with two ELISA protocols and serum dilutions, 1:10 and 1:50, was composed of 246 women (Figure I and Table 1, pages 53 and 54, respectively). The Bland-Altman analysis shows that there is a slightly difference between serum dilutions 1:10 and 1:50 with higher HPV16 antibody levels being detected in more diluted sera in both ELISA protocols (Figure 4, page 68). The upper and lower limits of agreement (95% CI) of the mean difference between both serum dilutions using the protocol with L1 VLPs were -0.164 (95% CI: -0.138 – -0.191) and -0.327 log units (95% CI: -0.354 – -0.300), respectively. The range between the upper [-0.273 (95% CI: -0.240 – -0.305)] and lower [-0.325 (95% CI: -0.358 – -0.203)] limits of agreement were larger when L1+L2 VLPs were used. The magnitude of differences between serum dilutions observed using L1 VLPs, and L1+L2 VLPs were -0.081 and -0.026 log units, respectively. Although they are below 1 log unit, the confidence intervals of the limits of agreement showed statistically significant results (the line of equality is not included in the interval). A greater dispersion of data points was observed using L1+L2 VLPs compared to L1 VLPs with 96.7% of the data points within the limits of agreement (mean ± 2SD) when the protocol was performed with VLPs composed by L1 only, and 93.1% with L1+L2. The regression line of the mean differences shows that the proportional difference between both serum dilutions is smaller when L1+L2 VLPs were used [R^2 = 0.098 (95%CI: 0.020 – 0.176)] in comparison to L1 VLPs [R^2 = 0.546 (95%CI: 0.445 – 0.647)]. The 95% CI of the determination coefficients (R^2) showed the statistical significance of these observations.



Protocol	Mean difference (SD)	Limits of agreement (95% CI)		β Coefficient (95% CI)	R^2 (95% CI)
		Upper	Lower		
A. L1 VLP (1:10 vs. 1:50)	- 0.081 (0.123)	- 0.164 (-0.138 – - 0.191)	- 0.327 (- 0.354 – - 0.300)	- 1.443 (- 1.609 – - 1.277)	0.546 (0.445 – 0.647)
B. L1+L2 VLP (1:10 vs. 1:50)	- 0.026 (0.150)	- 0.273 (- 0.240 – - 0.305)	- 0.325 (- 0.358 – - 0.293)	- 0.570 (- 0.787 – - 0.352)	0.098 (0.020 – 0.176)

Figure 4: Bland-Altman plot of differences between log-10 transformed HPV16 IgG NAR obtained by two serum dilutions versus the mean of the two measurements. They include regression lines and their confidence interval limits (grey bars). **A.** Virus-like particles (VLP) composed by L1 proteins. **B.** Virus-like particles (VLP) composed by L1 and L2 proteins. The magnitude of differences is represented by the gap between the Y axis corresponding to a zero difference (red dashed lines), and the parallel line to the X axis (mean). The 95% CI on the determination coefficient (R^2) was determined by bootstrapping.

Before testing the entire cohort (n=1,961) with the protocol that best captured the association between HPV16 seropositivity and HPV16 DNA positivity (L1+L2 VLP and the serum dilution 1:10) (Table 3, page 59), we analyzed the subset of women tested for both protocols and both serum dilutions (n=246) (Table I, page 70). We found that women infected with multiple HPV types were less susceptible to seroconversion in comparison to women infected with HPV16 only. Based on these analyses, we first observed that L1+L2 VLP was the best protocol to capture the association between HPV16 seropositivity and HPV16 DNA positivity. The best serum dilution to use with the L1+L2 VLPs was confirmed by the ROC curves. Dilution 1:10 presented the best area under ROC curve (ROC area=0.7330, 95% CI: 0.6465 – 0.8495) (Figure IV, page 57).

Table I. Linear regression between HPV16 seroreactivity and HPV status (single vs. multiple infection) at baseline in women tested by both protocols and serum dilutions

Protocol	Parameters	β coefficient (95% CI)
L1 VLP (1:10)	**Constant (β_0)**	-0.02 (-0.04 – 0.01)
	Else	Reference
	HPV16 single infection	0.11 (0.01 – 0.21)
	Multiple HPV infection with HPV16	0.05 (-0.07 – 0.17)
	R^2 (95% CI)	0.02 (-0.01 – 0.06)
L1 VLP (1:50)	**Constant (β_0)**	0.06 (0.02 – 0.10)
	Else	Reference
	HPV16 single infection	0.15 (0.00 – 0.29)
	Multiple HPV infection with HPV16	0.07 (-0.10 – 0.25)
	R^2 (95% CI)	0.02 (-0.02 – 0.05)
L1+L2 VLP (1:10)	**Constant (β_0)**	1.14 (-0.17 – -0.11)
	Else	Reference
	HPV16 single infection	0.27 (0.15 – 0.40)
	Multiple HPV infection with HPV16	0.17 (0.02 – 0.32)
	R^2 (95% CI)	0.09 (0.01 – 0.16)
L1+L2 VLP (1:50)	**Constant (β_0)**	-0.12 (-0.16 – -0.08)
	Else	Reference
	HPV16 single infection	0.34 (0.19 – 0.48)
	Multiple HPV infection with HPV16	0.15 (-0.03 – 0.33)
	R^2 (95% CI)	0.09 (0.01 – 0.17)

Protocols differ by the composition of the virus-like particles (L1 and L1+L2) and are based on two serum dilutions (1:10 and 1:50) (n=246)

Age was not an effect modifier as the introduction of an interaction term for age in our models was not statistically significant ($p > 0.05$). The analysis was done in the entire cohort (n=1,961). Detailed results of age as an effect modifier are presented below (Tables II and III,

pages 71 and 72, respectively). Table II shows the results by HPV status based on the phylogenetic relatedness to HPV16 HPV and Table III by type of infection (single vs. multiple).

Table II. Evaluation of age at enrollment as an effect modifier of the association between HPV16 seroreactivity and HPV DNA infection (HPV status by phylogenetic relatedness to HPV16) at baseline in the entire cohort

Parameters	ß Coefficients (95% CI)
Constant	-0.170 (-0.210 – -0.130)
Else	Reference
HPV16	0.325 (0.098 – 0.551)
HPV31/35	0.235 (-0.109 – 0.580)
HPV52/67/33/58	0.232 (-0.035 – 0.500)
Age at enrollment	0.003 (0.002 – 0.004)
HPV16 * Age	-0.006 (-0.013 – 0.001)
HPV31/35 * Age	-0.006 (-0.018 – 0.006)
HPV52/67/33/58 * Age	-0.005 (-0.013 – 0.003)
R^2 (95% CI)	0.002 (0.010 – 0.041)

Log10-transformed data obtained by the ELISA protocol using L1+L2 VLPs and serum dilution 1:10, n=1,961. CI: Confidence interval; CI on the determination coefficient (R2) was determined by bootstrapping. IgG NAR: Median (IQR) = 0.89 (0.62 – 1.23). * Interaction with age at enrollment.

Table III. Evaluation of age at enrollment as an effect modifier of the association between HPV16 seroreactivity and HPV DNA infection (single vs. multiple infection) at baseline in the entire cohort

Parameters	ß Coefficients (95% CI)
Constant	-0.159 (-0.198 – -0.119)
Else	Reference
HPV16 single infection	0.337 (0.066 – 0.607)
Multiple HPV infection with HPV16	0.369 (-0.122 – 0.860)
Age at enrollment	0.003 (0.001 – 0.004)
HPV16 single infection * Age	-0.006 (-0.014 – 0.002)
Multiple HPV infection with HPV16 * Age	-0.009 (-0.026 – 0.009)
R^2 (95% CI)	0.021 (0.007 – 0.035)

Log10-transformed data obtained by the ELISA protocol using L1+L2 VLPs and serum dilution 1:10, n=1,961. CI: Confidence interval; CI on the determination coefficient (R2) was determined by bootstrapping. IgG NAR: Median (IQR) = 0.89 (0.62 – 1.23) * Interaction with age at enrollment.

Figure 5 on page 73 shows the correlation between HPV16 seroreactivity and HPV16 viral load observed in the subset of 41 women with single HPV16 infection. Although the results have been mentioned in the manuscript, we included in this chapter the graphic representation of this analysis.

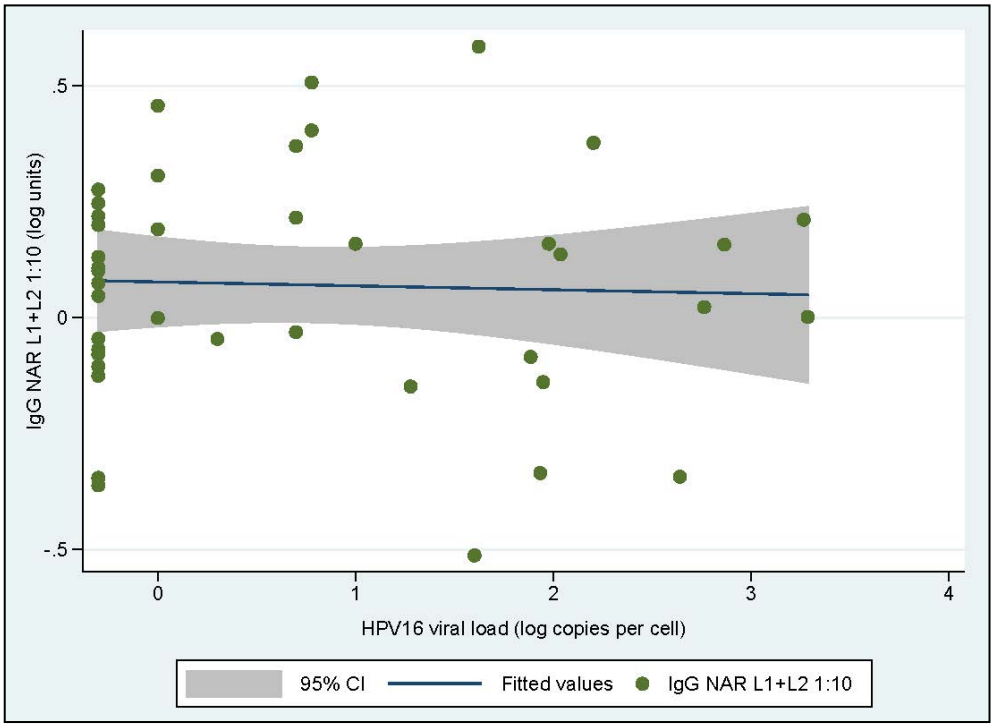


Figure 5: Correlation between log_{10}-transformed HPV16 IgG NAR and HPV16 viral load at baseline. Log_{10}-transformed HPV16 IgG NAR obtained by virus-like particles (VLP) composed by L1+L2, serum dilution 1:10. HPV16 viral load (log copies per cell) (n=41 women with single HPV16 infection). Pearson's correlation, r (95% CI) = -0.04 (-0.34 – 0.27), β coefficient (95% CI) = -0.01 (-0.08 – 0.06), R2 (95% CI) = 0.02 (-0.05 – 0.06). HPV16 viral load (copies/cell): median (interquartile range) = 2 (0.5 – 77.0), mean (standard deviation) = 154.0 (428.9), minimum=0.5, maximum=1,940.0. The 95% CI on the determination coefficient (R2) was determined by bootstrapping.

Chapter 5. Discussion

In this chapter we discuss our results in light of the literature, the limits and strengths of the study, and the potential threats for the internal and external validity.

5.1. Results in light of the literature

Although comparisons between serological assays have been done to measure antibody responses after HPV vaccination, studies evaluating the influence of the composition of the VLPs used as antigen in ELISA protocols to measure humoral immune response against naturally acquired HPV infection are lacking in the literature (34, 39, 163, 164). The Ludwig-McGill cohort study provides a unique opportunity to evaluate the association between HPV16 naturally acquired immunity and DNA positivity in a large sample size of women collected in the pre-vaccine era.

Our comparative analysis between different ELISA protocols showed, as expected, that both methods and both serum dilutions detected very low levels of HPV16 IgG antibodies following natural HPV infection compared to the level that could be detected after vaccination (9, 178). This weak natural immune response is probably related to the absence of viremia (179).

The L1 gene has the most conserved nucleotide sequence of the HPV genome. It can be aligned for all known papillomaviruses (59). Although L2 is not very immunogenic, antibodies against L1+L2 VLPs may block infection of a diverse range of other HPV genotypes in contrast to L1 VLPs (180). Technically, there is an increase in the yield of HPV16 VLPs when they are produced with L1 and L2 proteins compared to L1 only which is

an advantage for researchers planning to produce VLPs for their own serologic assays (133). Therefore, we investigated if the composition of the VLP used in the ELISA assays could affect the detection of HPV16 IgG antibodies. Our findings are supported by another study that compared Luminex multiplex assays performed with both VLP types. They showed that L1+L2 VLPs performed better for measuring HPV16 and 18 antibodies in large samples (39). Our results also showed a strong correlation between data obtained by serum dilutions 1:10 and 1:50 using both VLP types, and a moderate correlation between VLP types with the same serum dilution, suggesting that data obtained through different VLP types cannot be pooled in the analysis. Although results obtained with L1+L2 VLPs were more scattered around the regression line, they were also more stable between serum dilutions in comparison to L1 VLPs.

There are only a few studies in the literature that have focused on assay validation and optimization (44, 45, 47). In general, sensitivity of ELISA protocols using VLPs as antigens is between 50 to 60% with high specificity (>90%) (179). This variation in sensitivity may be due to different definitions of cut-off values between studies making the comparison between them even more difficult (179). Based on the literature and on our own experience, our team has proposed the use of normalized absorbance ratio (NAR) to circumvent the ELISA technical problems (intra- and inter-assay variability) that can affect the validity of seroreactivity (27, 30, 44-47). This method may provide a cost-effective alternative to keep the quality control of serological measurements in large epidemiological cohort studies (46). The GST-L1 antigens closely approximates the VLP-ELISA at a lower cut-off and may be an appropriate choice for studies aiming to assess population-level patterns in the epidemiology of cumulative infection with many HPV types (181).

The strength of the association between HPV16 antibodies and naturally acquired HPV DNA infection has been mostly investigated considering seropositivity as a predictor of HPV infection using logistic regression or generalized estimating equation (15, 25, 27-31, 33). We evaluated HPV infection as a predictor of seropositivity through linear regression in order to avoid using a cut-off for NARs. Residuals were randomly distributed which supported the application of this model in our analysis. Both linear and logistic regressions show the association between HPV16 seropositivity and HPV16 DNA positivity in cross-sectional studies (24).

HPV16 DNA positivity was considered as an independent determinant of HPV16 seropositivity in our study which is similar to the findings of others (13, 15, 27, 28, 30, 33). We have observed low degree of cross-reactivity for infections with other alpha 9 HPV types when we analyzed the entire cohort. Although not statistically significant, our results agree with others (24, 26, 182). The measured antibodies seem to be mainly type-specific. Although it is not clear in the literature what are the potential confounders of this association, we analyzed the impact of age as a potential confounder and effect modifier using the entire cohort (n=1,961) (25, 27, 28, 30, 38). Age was neither a strong confounder nor an effect modifier of this association.

It is also very common to find co-infections with multiple HPV types in many epidemiological studies (15, 30, 116). In the Ludwig-McGill cohort 12.3% of all study participants tested positive for multiple HPV types at baseline (183). The association measured for HPV16 with multiple types (co-infection) was similar to that for HPV16 single infection. It is possible that co-infections with multiple HPV types or high HPV16 viral load

reflect the inability of the immune system to respond to the viral infection (leading to low levels of antibodies), as others observed through logistic regression (15, 30). Researchers observed that the correlation between serology and HPV DNA status tends to be stronger among women infected with a single HPV type (median OR = 10.5, CI 95% = 2.4–48.4) than among women with multiple HPV infections (median OR = 4.6, CI 95% = 1.8–11.7) (15). In our models, neither HPV16 viral load nor co-infections with other types seem to influence the association between HPV16 seropositivity and HPV16 DNA positivity.

Serological assays to measure anti-HPV antibodies have a potential clinical utility to measure present and past exposure to HPV infection and could be used as a marker of HPV-associated disease (128). Although they cannot replace HPV DNA detection methods or cytological and histological examinations of the cervical cells, it can be an adjuvant test, especially in molecular epidemiology studies to investigate the natural history of HPV infection and cervical precancerous lesions (10, 184-186).

5.2. Limits and strengths of the study

The Ludwig-McGill cohort study is the largest epidemiologic investigation of the natural history of HPV infections ever done in the Brazilian population (165). This is a rare opportunity to investigate serological data from naturally acquired HPV infection in a vaccine era.

We tested samples from many women; however, participant's characteristics are not in line with our expectations for a region considered to be at high risk for cervical cancer. Most of them were on average 33 years old [median (IQR) = 32.0 (26.0 - 39.0)] and reported having had at most one lifetime sexual partner. More than 80% of them had no active HPV infection

(negative for HPV DNA) at the onset of the study. Despite the unfavorable characteristics of the study population, we could detect the presence of total HPV16 IgG antibodies in many of them, indicating cumulative exposure to the virus. We share the same technical limits of other serological studies. ELISA is the most common serological assay used in epidemiological studies; however, we do not have a gold standard method to compare our results. Although ELISA assays cannot differentiate between neutralizing and non-neutralizing antibodies, they do provide us information on cumulative exposure to the virus. One of the strengths of our study is the accessibility of serological data obtained by two ELISA protocols differing from each other by the composition of the VLP used as an antigen and based on two serum dilutions (1:10 and 1:50). VLPs were kindly provided by Dr. John Schiller from the National Institute of Health (USA), and Dr. Ian Frazer from the University of Queensland (Australia). Both are pioneers of the VLP production which gives us confidence in the quality of our antigens.

To our knowledge, this is the first study comparing ELISA protocols performed with two serum dilutions to evaluate the impact of the composition of the VLPs to detect HPV16 seroreactivity. Since the outcome (HPV16 seroreactivity) is kept as a continuous variable, we avoided having to establish a cut-off point for seropositivity which can vary between studies (179). Otherwise, without a cut-off point we cannot provide seroprevalence data in our study.

5.3. Potential threats to internal validity

Precision. Care was taken with the serum control used in the ELISA assays to control inter- and intra-assay variations. A pool of serum recovered from adult women was prepared in advance. In order to minimize measurement random errors, normalized absorbance ratios (NAR) were calculated by dividing the mean blank-subtracted optical densities by the

equivalent values of the control serum pool included in the same plate in triplicate, using different dilutions (46). One may suspect systematic errors (non-differential classification errors) since HPV DNA detection and seroreactivity data depends on several lab equipment's, especially PCR machines, pipettes, and the colorimetric plate reader which provides us the optical densities. Unfortunately, poor calibration of equipment cannot be analyzed statistically. If it happens, all the data may be off in the same direction, either too high or too low.

Bias. The participant's response rate was very high (70%), and they were randomly selected. In addition, women that tested for HPV16 seroreactivity were highly representative of the entire cohort. So, selection bias due to recruitment and sample testing is thus unlikely. We analyzed baseline data only, therefore we were not penalized for the loss of follow-up which increases the possibility of introducing selection bias in relation to possible differential losses depending on exposure and outcome. Information bias may not have happened because we did not deal with repeated measures; consequently, our analysis was not influenced either by the memory of the participants with respect to questionnaire responses or changes in their behavior during the study. Since we used linear regression to analyze our data, we did not have to define a cut-off point for HPV16 seropositivity, decreasing the possibility of misclassification linked to the outcome variable. The chance to have had differential classification error of the exposure is minimal since we had very few missing data related to the detection of HPV infection (14/1975 specimens) randomly distributed in the cohort. Missing data was probably due to the poor-quality of some DNA samples. In addition, samples for both DNA and serology tests were collected at the same visit and blindly tested for the exposure and outcome, decreasing the possibility of differential classification errors.

Confounding. We constructed a conceptual framework to illustrate our thoughts about the role of age on the HPV seroconversion followed to an exposure to HPV DNA infection (Figure 6, page 80).

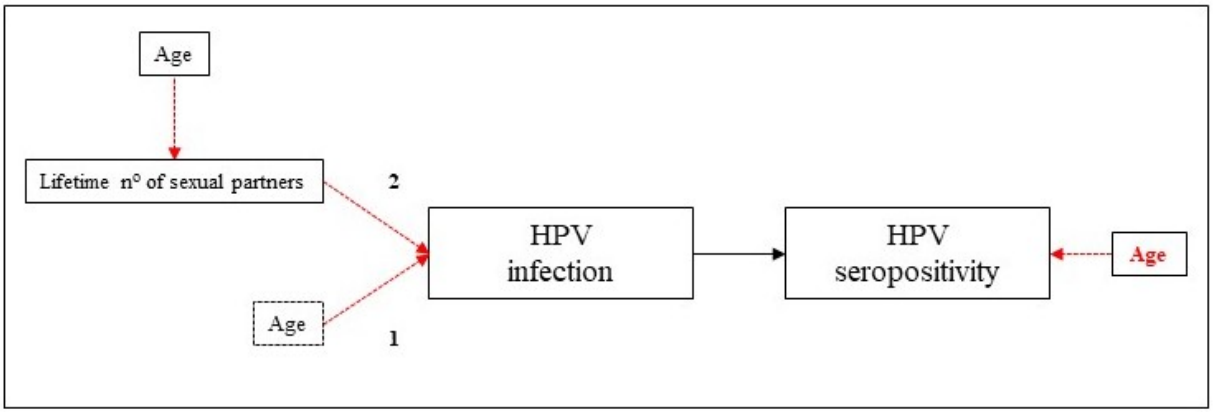


Figure 6: Conceptual framework. The role of age on the acquisition of an HPV DNA infection and seropositivity.

Although lifetime number of sexual partners seems to be strongly associated with HR-HPV DNA and seroprevalence, we ruled out the possibility of considering this variable as a confounder in this study, since most cohort participants reported at enrolment having had at most only one lifetime sexual partner. It is easy to understand that there is a reduction of immune responsiveness in the elderly (187). However, it is difficult to find a biological explanation to justify changes in the risk to acquire an HPV infection as we get older. We understand that it is very difficult to acquire an HPV infection without having sexual activity. Virgins have less chance to acquire an HPV infection, no matter their age (188-191). Consequently, there is no antibody production without having had an HPV infection (present or past). Regardless, several studies have found similar results reporting age as a statistically significant determinant for the acquisition of an HPV infection (see chapter 1). In a study done

with sexually active women of all ages, HPV16 seroprevalence tended to remain elevated compared to DNA positivity (33). HPV16 seroprevalence reached its highest peak at 25–34 years of age. Although seropositivity appeared to decline slightly with age after its peak, HPV16 seroprevalence always remained elevated above the level seen in women less than 25 years old. In contrast, HPV16 DNA positivity peaked in women less than 25 years old and declined with increasing age. They also observed a slight secondary increase in DNA prevalence in women older than 55 years old. However, there are controversies in the literature regarding all these findings (28, 38, 192). We have two hypotheses for the role of age in the association between HPV infection and seropositivity. The first one, less likely to happen, is that age is considered an independent factor directly associated to the acquisition of an HPV infection. The second one is the most biologically coherent hypothesis where age is considered a proxy of the lifetime number of sexual partner in its association with the acquisition of an HPV infection. In our opinion, young women tend to have more sexual partners than older women until they get divorced/widowed and start again having more sexual partners; consequently, they have more chance to acquire a new HPV infection. Although believing that age is not a direct determinant of the acquisition of an HPV infection, we adjusted our analysis by the age of the participants at the enrollment of the study to verify any changes in the β coefficients of the linear regression analysis. In this study, adjustment for age marginally changed the association between HPV16 seropositivity and HPV DNA infection confirming our hypothesis that age cannot be considered a confounder in this relationship. We also observed that age was not an effect modifier of the association.

5.4. Potential threats to external validity

Difficulty of generalizing beyond people. The participants may be different from the non-participants due to their socioeconomic status (low-income women) or because they are more concerned with their health.

Difficulty of generalizing beyond location. The study was conducted in Sao Paulo, Brazil. However, the variables under investigation in this study (HPV DNA positivity and HPV16 seroreactivity) are unlikely to be affected by the genetics or lifestyle (culture) of the participants. Therefore, it is unlikely that we will have problems in extrapolating our findings to other populations. However, caution was taken to discuss our data. We compared our results with results all over the world. However, caution was taken to discuss our data. We compared our results with results all over the world.

Difficulty of generalizing beyond time. The participants were selected from 1993 to 1997, and their follow-up finished more than 10 years ago. Caution was thus taken while interpreting our results in 2018. The literature review was done with no restriction of date.

Conclusion

We observed an association between HPV16 seropositivity and HPV16 DNA positivity do not seem to be affected by co-infections or viral load (as per the protocol used in this study). Other HPV types, even those related to HPV16, such as HPV31, 35, 52, 67, 33 or 58 HPV seem to not be associated with HPV16 IgG antibodies. The protocol using L1+L2 VLPs and serum dilution 1:10 better capture the association between HPV16 seropositivity and HPV16 DNA positivity.

Several questions remain about HPV serology. Despite the hard work of many researchers to identify the determinants of HPV seroreactivity, we still have a lot of controversies in the literature (13, 24, 27, 29, 30, 33, 38, 108, 154-161). We do not know why not all women seroconvert after prior exposure to HPV. It may be due to methodological issues (study design, assay, antigen, antibody, etc.) or a failure still not identified in their immune system. It has been reported that the median time from HPV16 DNA detection to seroconversion varies from 6-12 months (10, 125); but we do not know if it could vary according to the characteristics of the population under investigation. The duration of natural immunity is also unclear (13, 15, 17). In addition, studies showing the dynamics of HPV antibodies are missing in the literature. It is unclear if the naturally acquired immune response to HPV infection can effectively protect against reinfection, reactivation of a latent infection, or cervical precancerous lesions, and if it can clear an HPV infection (14, 16, 18-23, 193). Therefore, further studies are needed to investigate the naturally acquired immunity over time which represents a huge challenge in the HPV vaccine era.

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Appendix I. Inclusion and exclusion criteria for the summary table of the literature review

Inclusion criteria
1. Studies done in healthy women from the general-population; 2. Women with ≥18 years of age must be included in the cohort; 3. Studies done with children, men, and women were included if data were stratified by sex and/or age; 4. Baseline data for HPV16 DNA, seroprevalence and/or determinants of seroreactivity; 5. Data from natural acquired cervical HPV infection; 6. Studies evaluating HPV16 IgG antibodies only; 7. Studies providing HPV16 seroprevalence by HPV16 DNA status; 8. Studies evaluating seroreactivity against L1 and/or L1+L2 capsid proteins; 9. Articles published in English, French, and Portuguese; 10. Major articles only; 11. Full article available.
Exclusion criteria
1. Studies done animals; 2. Studies done with minorities (eg., patients diagnosed with cervical precancerous lesions and/or cervical cancer, pregnant women, HIV infected women, virgins, etc.); 3. Studies exclusively done with children and/or adolescents (<17 years of age); 4. Studies done with children, men, and women which data were not stratified by sex and/or age; 5. Studies evaluating HPV16 seropositivity as a determinant of reinfection (repeated measures); 6. Studies evaluating seroincidence/seroconversion, and seropersistence.

7. Studies done with vaccinated subjects;

8. HPV16 DNA and/or seropositivity data provided exclusively in combination with other HPV types (HR-types);

9. HPV16 DNA and/or seropositivity data from anatomical regions other than the cervix;

10. Studies providing HPV16 serology data without mentioning the HPV16 DNA status of the subjects;

11. Studies evaluating types of antibodies other than total HPV16 IgG antibodies (e.g., IgA, IgM, subclasses of IgG, and neutralizing antibodies);

12. Studies that did not specify, directly or indirectly (via reference), the type of antibody evaluated;

13. Studies on IgG antibodies other than L1 and L2 (e.g., E6, E7, etc.);

14. Studies providing odds ratios without proof of statistical significance (e.g., 95% confidence interval or p value);

15. Reviews and communication reports (if relevant are cited in the body of the dissertation).

Note. The research was done in PubMed using several combinations of key words and restricted to human subjects. There was no date or study design restriction. The selection of the articles was made by applying the inclusion and exclusion criteria in four steps: (1) Title relevance; (2) Abstracts evaluation; (3) Full article content, and (4) Evaluation of the references of selected articles.

Appendix II. Summary table of the literature review

The association between HPV16 serology and HPV DNA infection in transversal analysis (baseline data), and the determinants of HPV16 serology

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
Carter et al. (1996) USA	Baseline data from a longitudinal study 1990-1995 n=294 (18-20)	52.6 vs. 6.9 (p<0.001) ELISA (L1 VLP)	There is an association between HPV16 DNA infection and HPV16 serology (Fisher's exact test: p<0.001) <u>Determinants of HPV16 serology</u> Nº of lifetime sexual partners >10: 23.1% (3/13) (Chi-squared test: p<0.01)	-
			Women who seroconverted were 5.7 times (95% confidence interval = 2.4-13.4) more likely to have precancerous lesions associated with the detection of HPV16 DNA than were women who did not seroconvert.	
Nonnenmacher et al. (2003) Brazil	Cross-sectional July-Aug. 1994 n=976 (15-70)	44.9 vs. 35.3 ELISA (L1 VLP)	Unconditional logistic regression: There is an association between HPV16 DNA infection and HPV16 serology: 1.61 (1.20–2.20)	Unconditional logistic regression: final model defined by stepwise backwards method: p value for removal=0.15 and for entry=0.10 Adjusted for: age, and HPV16 DNA+ Age (years) ≤24: reference 25-34:1.56 (1.00-2.50) 35-49: 1.87 (1.20-2.90) 50+: 1.37 (0.80-2.30) Lifetime nº sexual partners 1: reference 2: 1.89 (1.40-2.60) 3: 1.82 (1.20-2.80) 4+: 2.95 (1.90-4.50) Cytological diagnosis of SILs in HPV16 seropositive women Age-adjusted: 2.07 (1.0–4.5)
			Seropositivity to HPV 16 and HPV 6/11/16/18 antigens seem to be better markers of past sexual activity than current HPV infection, and humoral response to HPV16 or HPV6/11/16/18 may not be a strong indicator of cervical lesions in populations at low risk for cervical lesions.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
Wang et al. (2003) Costa Rica	Cross-sectional 1993-1994 HPV16: n=9949 HPV18: n=9928 HPV31: n=9932 HPV45: n=3019 CIN III/cancer: n=107 (18-97)	45.0 In women infected with HPV31: 2.0 (1.6 –2.6) In women infected with HPV18: 1.9 (1.5 – 2.5) ELISA (L1 VLP)	Logistic regression: There is an association between HPV16 DNA infection and HPV16 serology: 4.50 (3.60–5.60) For all four HPV types measured, the magnitude of the association was highest for each HPV serotype and DNA of the same type. Sero- and HPV prevalence varied with age	Logistic regression: Adjusted for: age, and nº of sexual partners in the past year Lifetime nº sexual partners (Data for HPV16) 1: reference 2-3: 2.10 (1.80 –2.30) 4+: 3.1 (2.60 –3.70) Lifetime number of sexual partners was the key determinant of seropositivity independent of DNA status and age. Oral Contraceptive use Never: reference Former: 1.30 (1.10 – 1.50) Current: 1.5 (1.20 – 1.80) Adjusted for: age Diagnosis of CIN III/cancer Sero-/DNA-: reference Sero+/DNA+: 34.70 (19.70 – 61.00) Sero-/DNA+: 39.90 (24.10 – 66.20) Sero+/DNA-: 2.00 (1.10 – 3.70)
			DNA-positive and seropositive women showed the highest risk for concurrent CIN III/cancer, followed by DNA-positive, seronegative women.	
Faust et al. (2013) Slovenia	Cross-sectional Dec. 2009-Aug. 2010 n=3,291 (20-64)	56.7 vs 23.9 Luminex (L1+L2 VLP)	Logistic regression: There is an association between HPV16 DNA infection and HPV16 serology: 4.31 (2.27–8.21) Sero+/multiple type DNA+: 4.26 (2.62–6.93) HPV types evaluated: 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 68, and 73) The correlation between serology and HPV DNA status tended to be stronger among women infected with single HPV type (median OR = 10.5, CI 95% = 2.4–48.4) than among women with multiple HPV infections (median OR = 4.6, CI 95% = 1.8–11.7)	-
			A multiplexed HPV PsV-Luminex assay has been developed and validated to correlate with natural HPV infection for 13 HPV types, thus enabling more comprehensive studies in HPV epidemiology and vaccine research.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
Dondog et al. (2008) Mongolia	Cross-sectional Sept.-Nov. 2005 n=969 (15-59)	33.9 vs. 22.3 Luminex (GST-L1)	-	Logistic regression: Adjusted for: age, and lifetime nº of sexual partners There is an association between HPV16 DNA infection and HPV16 serology: (Chi-squared test, p=0.046) Age (years) <25: reference 25-29: 0.90 (0.50-1.40) 30-34: 1.00 (0.60-1.60) 35-39: 1.40 (0.90-2.20) 40-44: 1.20 (0.70-1.90) 45-49: 1.70 (1.00-2.60) 50+:2.00 (1.20-3.10) Lifetime nº sexual partners 1: reference 2: 1.10 (0.80-1.50) 3: 1.30 (0.90-1.90) 4+: 1.50 (1.00-2.10) Husbands' extramarital sexual relationships Never: reference Ever: 1.40 (1.00-2.00) Induced abortion Never: reference Ever: 1.40 (1.00-1.90)
			The proportion of women positive for HPV16 DNA or antibodies was similar among women ages <35 years (27.6%) and ages ≥35 years (26.6%). However, older women were less likely to be HPV16 DNA positive (with or without corresponding antibodies) and more likely to be HPV16 seropositive only. Lifetime number of sexual partners and induced abortions were shown to be directly associated with HPV DNA and/or seroprevalence.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
De Araujo-Souza et al. (2014) Brazil	Baseline data from a longitudinal study 1993-1997 n=2,049 (18-60)	20.7 ELISA (L1 and L1+L2 VLP)	Unconditional logistic regression: Age (years) <25: reference 25-34:1.33 (0.97-1.84) 35-44: 1.55 (1.11-2.15) 45+: 2.11 (1.42-3.12) Lifetime nº sexual partners 0-1: reference 2-3:1.70 (1.32-2.19) 4-5: 2.71 (1.97-3.32) 6+: 2.34 (1.59-3.44) Age at first intercourse (years) 20-50: reference 18-19:1.30 (0.93-1.79) 16-17: 1.29 (0.94-1.76) ≤15: 1.68 (1.25-2.27) HPV16 DNA+ No: reference Yes: 3.60 (2.11-6.13) HPV16 DNA+ (single vs multiple infection) Negative: reference Single: 3.80 (2.01-7.19) Multiple: 3.60 (2.11-6.13) HPV16 viral load (copies/cell) Negative: reference <1: 2.91 (1.16-7.28) 1-100:4.33 (1.96-9.58) ≥100: 3.43 (1.14-10.30) Non HPV16 alpha PV-9 No: reference Yes: 2.00 (1.20-3.21) Any HPV No: reference Yes: 1.40 (1.07-1.83) Any HR-HPV No: reference Yes: 1.60 (1.16-2.19)	Unconditional logistic regression: Adjusted for: age, and HPV16 DNA+ Age (years) <25: reference 25-34: 1.38 (0.97-1.84) 35-44: 1.60 (1.15-2.23) 45+: 2.16 (1.45-3.22) Lifetime nº sexual partners 0-1: reference 2-3: 1.70 (1.32-2.20) 4-5: 2.56 (1.97-3.53) 6+: 2.29 (1.55-3.37) Age at first intercourse (years) 20-50: reference 18-19: 1.52 (1.08-2.13) 16-17: 1.60 (1.15-2.23) ≤15: 2.18 (1.59-3.00) Frequency of sex 0-1 times: reference 2-3: 0.95 (0.75 – 1.22) 4-5:1.53 (1.04 – 2.26) 6+: 1.77 (1.07 – 2.92) Duration of smoking Never: reference ≤10 years: 0.81 (0.61 – 1.07) 11+ years: 0.62 (0.57 – 0.98) <u>Age-adjusted:</u> HPV16 DNA+ No: reference Yes: 3.86 (2.23-6.59) HPV16 DNA+ (single vs multiple infection) Negative: reference Single: 3.93 (2.07-7.48) Multiple: 3.86 (2.23-6.59) HPV16 viral load (copies/cell) Negative: reference <1: 3.10 (1.23-7.79) 1-100: 4.64 (2.09-10.30) ≥100: 3.73 (1.24-11.20)

				Non HPV16 alpha PV-9 No: reference Yes: 2.17 (1.32-3.56) Any HPV No: reference Yes: 1.52 (1.15-1.83) Any HR-HPV No: reference Yes: 1.73 (1.25-2.39)
			In multivariate analysis, seroreactivity was positively correlated with age, lifetime number of sexual partners, frequency of sex, and HPV16 viral load, and negatively associated with duration of smoking. In summary, HPV16 seroreactivity is determined by factors that reflect viral exposure.	
Triglav et al. (2017) Slovenia	Baseline data from a longitudinal study Dec. 2009-Aug. 2010 HPV16 DNA-: n=2111 HPV16 DNA+: n=88 (20-64)	55.7 vs 23.2 ($p<0.01$) Luminex (VLP)	Unconditional logistic regression: There is an association between HPV16 DNA infection and HPV16 serology: 4.2 (2.70-6.40)	-
			Naturally acquired anti-HPV16 serum antibodies appeared to protect against anogenital HPV16 infection, but this association was at least partially confounded by age. Baseline anti-HPV16 serum antibodies did not influence persistence/clearance of HPV16 infection at follow-up.	
Michael et al. (2008) [c] Germany	Cross-sectional Oct. 1985-Jan. 1989 Male: n=758 Female: n=1,039 (1-82)	10.9 (women > 14 years old) 0.5 (children) Serological data was stratified by age and sex Luminex (GST-L1)	Highly significant seroprevalence increases from children to younger adults (15–34 years) (but not from younger to older adults >34 years) (Fisher's exact test, $p<0.0001$). The antibody prevalence to HPV16, peaked between 25 and 34 years.	Age standardization was applied, but changed seroprevalence estimates only marginally.
			We provide evidence for different age- and sex-dependent seroprevalence patterns of phylogenetically related HPV: antibodies to cutaneous mu and nu PV appear early in life, those to mucosal alpha PV after puberty, and those to beta and gamma skin PV accumulate in adulthood.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
Castro et al. (2014) Chile	Baseline data from a longitudinal study 2001 n=1,021 (15-86)	18.5 (95% CI: 16.2-21.0) Luminex (GST-L1)	GEE: There is an association between HPV16 DNA infection and HPV16 serology: 1.3 (0.5-3.4) Seropositivity for each HPV type (16, 18, 31, 33, 35, 45, 52, and 58) tended to be higher among DNA-positive infections with the same type, reaching statistical significance only when all infections were analyzed together: positive to the same HR-HPV type: 1.7 (1.1-2.5)	GEE: Variables with $p \leq 0.2$ in univariate models were included in the multivariate model. Adjusted for: age, age at first intercourse, lifetime nº of sexual partners, smoking, cervical HPV DNA Age (p trend<0.001) 15-20: reference 21-30: 0.70 (0.40-1.22) 31-40: 1.24 (0.73-2.11) 41-50: 1.09 (0.59-1.69) 51-60: 2.15 (1.15-3.32) ≥ 61: 2.16 (1.17-3.47) Age at first intercourse (p trend<0.001) 15: reference 16-17: 0.79 (0.57-1.08) 18-19: 0.66 (0.47-0.92) ≥ 20: 0.53 (0.38-0.74) Lifetime nº of sexual partners 1: reference ≥2: 1.30 (1.01-1.67) Cervical HPV DNA Negative: reference Positive: 1.48 (0.86 – 2.56)
			HPV seroprevalence studies are a useful tool for learning about the dynamics of HPV infection in a community. This study contributes to understanding the natural history of HPV infection and provides a baseline assessment before the incorporation of HPV vaccination into a national program.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
Nonnenmacher et al. (1996) Greenland (i) Denmark (ii)	Cross-sectional 1993 n=153 (i) n=124 (ii) (21-33)	<u>Greenland</u> 33.0 vs 58.0 (Fisher's exact test, p=0.18). <u>Denmark</u> 38.0 vs 38.0 (p >0.05) ELISA (L1 and L1+L2 VLP)	<u>Greenland</u> <u>Determinants of HPV16 serology</u> Age (years) (Chi-squared trend, p>0.1) 35+: reference 30-34: 0.30 (0.07-1.30) 25-29: 0.30 (0.07-1.00) 20-24: 0.40 (0.09-1.60) <20: 0.20 (0.04-1.10) Lifetime nº sexual partners (Chi-squared trend, p>0.25) 35+: reference 25-34: 1.50 (0.90-2.60) 15-24: 0.80 (0.30-1.90) 5-14: 1.10 (0.40-2.70) <5: 0.20 (0.02-1.90) <u>Denmark</u> <u>Determinants of HPV16 serology</u> (Chi-squared trend, p<0.001) Age (years) 35+: reference 30-34: 0.40 (0.10-1.10) 25-29: 0.20 (0.05-0.60) 20-24: 0.10 (0.03-0.40) <20: 0.10 (0.01-0.60) Lifetime nº sexual partners (Chi-squared trend, p<0.001) 35+: reference 25-34: 0.40 (0.10-1.30) 15-24: 0.30 (0.10-0.90) 5-14: 0.20 (0.05-0.50) <5: 0.10 (0.02-0.60)	-
			Since genital HPV DNA prevalence decreased with increased cumulative HPV exposure in the 2 high risk groups examined, we conclude that HPV DNA is not a valid marker for comparing relative exposure to HPV in high- and low-risk populations. Seroreactivity using the VLP ELISA appears to reflect relative cumulative exposure to the virus more closely, although in very high-risk cohorts, such as the Greenlandic one examined here, there may be a plateau at high levels of exposure.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
Porras et al. (2010) Costa Rica	Baseline data from the Costa Rica HPV Vaccine Trial (pre-vaccination) Jun. 2004- Dec. 2005 n=646 (18-25)	63.0 ELISA (L1 VLP)	Unconditional logistic regression: Frequency sexual intercourse, month (ANOVA, p trend=0.27) ≤1: reference 2-3:1.92 (1.03-3.57) 4-9: 1.79 (1.07-2.99) 10+: 1.96 (1.14-3.36) (ANOVA, p trend=0.27) Lifetime nº sexual partners (ANOVA, p trend=0.04) 1: reference 2: 1.23 (0.75-2.03) 3+: 1.62 (1.02-2.59) Use of hormonal contraceptives Never: reference In the past: 1.33 (0.76-2.33) Current use of oral contraceptive: 1.88 (1.14-3.09) Current injectable: 3.38 (1.39-8.23) Use of condom last sexual intercourse No: reference Yes: 0.54 (0.37-0.81) Cytology/Viral load Normal/Low viral load: reference LSIL/Low viral load: 2.12 (0.84-5.34) HSIL/Low viral load: 2.79 (0.98-7.93) Normal/High viral load: 2.22 (1.28-3.85) LSIL/High viral load: 1.63 (0.97-2.75) HSIL/High viral load: 2.50 (1.30-4.81)	Unconditional logistic regression: Of particular interest were variables that could be markers of timing of HPV infection (time since sexual debut and time with most recent partner) or of amount/load of exposure (number of sexual partners, viral load by HC2, cytologic finding, hormonal contraception, and condom use). Possible confounding factors were explored, and a final model was built for each characteristic of interest adjusting for all other variables that changed the crude OR estimates by 15% or more. <u>Adjusted for the use of hormonal contraceptive</u> Frequency sexual intercourse ≤1: reference 2-3: 1.85 (0.98-3.46) 4-9: 1.55 (0.91-2.65) 10+: 1.57 (0.89-2.77) <u>Adjusted data for time with most recent partner</u> Lifetime nº sexual partners 1: reference 2: 1.48 (0.87-2.50) 3+: 1.96 (1.19-3.25) <u>Adjusted data for use of hormonal contraceptive</u> Use of condom last sexual intercourse No: reference Yes: 0.66 (0.42-1.03)
			Factors associated with sustained HPV exposure (abnormal cytology, elevated HPV viral load, increasing lifetime partners) were predictive of HPV16 seropositivity. Hormonal contraceptive use was associated with seropositivity suggesting an effect of hormones on immune responses to HPV.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
			-	-
Carter et al. (2000) USA	Baseline data from a longitudinal study 1990-1998 n=588 (18-20)	54.2 ELISA (VLP)	Seroconversion study which provides the seroprevalence of HPV16 in women infected with the same HPV type at baseline. Antibody responses to each type were heterogeneous, but several type-specific differences were found: seroconversion for HPV16 occurred most frequently between 6 and 12 months of DNA detection, but seroconversion for HPV6 coincided with DNA detection. Additionally, antibody responses to HPV16 and 18 were significantly more likely to persist during follow-up than were antibodies to HPV6.	
Olsen et al. (1997) Norway	Case-Control 1991-1992 Normal cytology: n=208 HPV signs in cytology: n=20 CIN II/II/Cancer: n=6 (20-44)	17.6 ELISA (VLP)	The proportion of women with antibody response above 0-100 (cut-off) was significantly lower in the category with normal cytology than in the categories of cytological features of HPV or CIN (Chi-squared: 6.8, p = 0.03).	Multivariate logistic regression: Adjusted for: age, age at first sexual intercourse; and number of sexual partners. Neither age nor age at first sexual intercourse was associated with HPV16 antibodies Nº of sexual partners (p trend<0.01) 0-1: reference 2-3: 2.90 (0.30-30.80) 4-5: 13.10 (1.50-110.80) 6-10: 8.20 (1.00-69.60) 10+: 10.50 (1.20-94.00)
			Seropositivity to HPV16 capsids is positively associated with the number of sexual partners, suggesting that HPV16 is predominantly sexually transmitted. The fact that serology had a stronger association with number of sexual partners than viral DNA suggests that seroreactivity is a better measure of lifetime history of HPV infection.	

Authors (year)/ Location	Study design/Enrollment/ Sample size/(Age) [a]	HPV16 seroprevalence (HPV16 DNA positive vs. negative) [b] (%)/Assay	Determinants of HPV16 serology: OR (95% IC) or p value/Conclusion	
			Unadjusted analysis	Adjusted analysis
Coseo et al. (2010) Costa Rica	Baseline data from the Costa Rica HPV Vaccine Trial (pre-vaccination) Jun. 2004- Dec. 2005 n=5,871 (18-25)	63.0 vs 27.9 ELISA (VLP)	Univariate unconditional logistic regression: Age (years) 18-19: reference 20-21:1.30 (1.10-1.53) 22-23: 1.57 (1.33-1.84) 24-25: 1.70 (1.45-2.00) Years since sexual debut 0-1: reference 2-3:1.66 (1.32-2.09) 4-5: 2.19 (1.75-2.75) 6-7: 2.58 (2.04-3.25) 8+: 3.23 (2.56-4.07) Marital status Married: reference Single:1.12 (1.00-1.25) Separated/Divorced/Widowed: 1.55 (1.15-2.09) Lifetime nº sexual partners 1: reference 2-3: 2.04 (1.79-2.32) 4+: 3.52 (3.00-4.14) Nº of pregnancies 0: reference 1:1.36 (1.20-1.55) 2+: 1.81 (1.57-2.09) Hormonal contraceptives Neither: reference OC:1.33 (1.12-1.57) Inj. C: 1.84 (1.42-2.40) OC + Inj. C: 1.67 (1.41-1.99) Smoking Never: reference Former:1.52 (1.23-1.89) Current: 1.73 (1.45-2.08) Current and/or past STIs No: reference Yes:1.86 (1.61-2.14) HC2/cytology result HC2-/normal: reference HC2+/normal: 2.05 (1.78-2.36)	Multivariate unconditional logistic regression: p value for entry <0.10 in a univariate model Adjusted for: years since sexual debut, lifetime nº of sexual partners, nº of pregnancies, hormonal contraceptive use, condom use, smoking history, current and/or past STIs, HC2/cytology result. Years since sexual debut 0-1: reference 2-3: 1.39 (1.09-1.79) 4-5: 1.55 (1.20-2.02) 6-7: 1.69 (1.24-2.21) 8+: 1.82 (1.35-2.46) Lifetime nº sexual partners 1: reference 2-3: 1.53 (1.38-1.77) 4+: 2.19 (1.81-2.65) Nº of pregnancies 0: reference 1: 1.22 (1.03-1.44) 2+: 1.37 (1.11-1.69) Hormonal contraceptives Neither: reference OC: 1.11 (0.92-1.34) Inj. C: 1.44 (1.07-1.93) OC + Inj. C: 1.15 (0.93-1.42) Smoking Never: reference Former: 1.21 (0.95-1.54) Current: 1.29 (1.05-1.57) Current and/or past STIs No: reference Yes: 1.44 (1.23-1.67) HC2/cytology result HC2-/normal: reference HC2+/normal: 1.83 (1.58-2.14) HC2+/mild alterations: 2.07 (1.70-2.53) HC2+/mild to severe alterations: 3.21 (2.38-4.34)

			HC2+/mild alterations: 2.17 (1.80-2.62) HC2+/mild to severe alterations: 3.61 (2.72-4.79)	*Linear regression models with log-transformed continuous antibodies titers results were similar to dichotomous models (data not shown)
			There was no evidence of assay cross-reactivity as HPV16 seroprevalence was similar (approximately 34%) among women singly infected with genetically and nongenetically related species (α9 and non-α9). The increasing seroprevalence observed with time since first sex suggests that HPV serology is a cumulative marker of HPV exposure. However, many DNA infected women were seronegative; thus, serology is an imperfect measure of past exposure to cervical HPV, at best. Additionally, we found no evidence of assay cross-reactivity.	
Liu et al. (2016) [c] China	Baseline data from a longitudinal study 2007-2009 Men: n=1,603 Women: n=2,187 (25-65)	0.3 vs 9.2 Luminex (GST-L1)	Logistic regression: Data were grouped by oncogenic types (HPV16, 18, 45, 52, and 58). HPV16 only not showed. Few subjects were dually positive to HPV DNA and serum antibodies for any HPV (3.1% of women). Positivity for oncogenic HPV DNA and seropositive for the same type 1.89 (1.00–3.57) Among 762 couples, the presence of HPV DNA and/or antibodies in one partner was positively associated with the identical HPV type in the other partner. Oncogenic types: 1.56 (1.10–2.21)	Multivariate logistic regression: Data were grouped by oncogenic types (HPV16, 18, 45, 52, and 58). HPV16 only not showed. Adjusted for: age, cigarette smoking, alcohol consumption, lifetime, nº of sexual partners. Positivity for oncogenic HPV DNA and seropositive for the same type 1.91 (1.01–3.60) Among 762 couples, the presence of HPV DNA and/or antibodies in one partner was positively associated with the identical HPV type in the other partner. Oncogenic types: 1.55 (1.09–2.20)
			These findings may reflect a site-specific natural course of HPV infection.	

Abbreviation. OR, odds ratio; CI, 95% Confidence interval; GEE, generalized estimating equation; PRR, prevalence rate ratio; USA, United States of America; GST-L1, glutathione S-transferase-L1-flag-fusion proteins; ELISA, Enzyme-linked immunosorbent assay; VLP, virus-like particle; PV, papillomavirus; RCT, Randomized clinical trial; STIs, sexually transmitted infections; HC2, Hybrid capture 2; OC, oral contraceptive; Inj. C., injectable contraceptive; HR-HPV, High-risk HPV types; HSV2, herpes simplex virus type 2; LSIL, low-grade squamous intraepithelial lesions; HSIL, high grade squamous intraepithelial lesion; CIN II and III, Cervical intraepithelial neoplasia of grade II and III, respectively.

[a] Sample size is presented as number of subjects and age in years.

[b] Data from women with vs. without HPV16 DNA infection, if not, data is from women with HPV16 DNA infection only. 95% CI or p-value are provided whenever informed.

[c] Results are from women only.

Appendix III. Codebook of the Ludwig-McGill study baseline questionnaire

Brazilian Study, Questionnaire 1			
Question		**Code**	**Descriptor**
Number	**Descriptor**		
Date	Interview	Date	
No	Study number	Num	
MEVNC	Hospital number	Num	
NOME	Name	Initial	
NASCEU	Birth date	Date	
ANOS	Age	Num	
5	Ethnic group	1	White
		2	"Mulata"
		3	Black
		4	Asian
		5	Native Indian
6	Marital status	1	Single
		2	Married
		3	Widowed
		4	Separated
		5	Unmarried, but living with partner
7	Occupation for the 10 past years	N-Num	
8	Level of schooling	1	Illiterate
		2	Elementary incomplete
		3	Elementary completed
		4	Secondary incomplete
		5	Secondary completed
		6	College-Technical-professional
		7	University
9	Religion	1	Catholic
		2	"Crente"
		3	Protestant
		4	Jewish
		5	Spiritism
		6	Umbanda
		7	Other
		8	None
		9	?
9B	Religion for those coded 7 at Q.9	N-Num	

10	Number of person living with her including herself	Num	
11	Family income	Num	Questionnaire shows cruzeiros
12a	Household goods: Refrigerator	1	Yes
		2	No
12b	Household goods: Color TV	1	Yes
		2	No
12c	Household goods: Phone	1	Yes
		2	No
12d	Household goods: Videotape	1	Yes
		2	No
12e	Household goods: Car	1	Yes
		2	No
12f	Household goods: Another car	1	Yes
		2	No
13	District (where she lives)	Non-num	
14	Number of years (that she lives at this place)	Num	
15a	Birth place (city)	Non-num	
15b	Birth place (State)	Non-num	
16	Type of birthplace	1	Rural
		2	Urban
		3	Suburb
		8	Don't know

17a	Where she spent the major part of their life after 12 years old (city)	Non-num	
17b	Where she spent the major part of her life after 12 years old (state)	Non-num	
18	Type of era where she spent the major part of her life	1	Rural
		2	Urban
		3	Suburb
		4	Don't know
19	Ever smoked	1	Yes
		2	No
20	Number of cigarettes smoked in average by day (commercial cigarettes)	1	No more than 1
		2	2 to 5
		3	6 to 10
		4	11 to 20
		5	More than 20
		6	More than 40 (2 packs)
21	Type of cigarettes (commercial cigarettes)	1	Only with filter
22	Age she started smoking (commercial cigarettes)	Num	
23	(If she still smoking, number of years she smokes) (commercial cigarettes)	Num	
24	If she quitted smoking, number of years she had smoked (commercial cigarettes)	Num	

25	Number of cigarettes smoked in average by day (homemade cigarettes; stronger)	1	No more than 1
		2	2 to 5
		3	6 to 10
		4	11 to 20
		5	More than 20
		6	More than 40 (2 packs)
26	Age she started smoking (homemade cigarettes)	Num	
27	If she still smoking, number of years she smokes (homemade cigarettes)	Num	
28	If she quitted smoking, number of years she had smoked (homemade cigarettes)	Num	
29	Age she quitted smoking	Num	
30	Ever smoked cigars or pipe	1	Yes
		2	No
31	Ever drank alcohol occasionally	1	Yes
		2	Never
32	Ever drank beer	1	No / occasionally
		2	No more than one glass per week
		3	2-5 per week
		4	6-10 per week
		5	11-30 per week
		6	More than 30 per week
33	Ever drank wine	1	No / occasionally
		2	No more than one glass per week
		3	2-5 per week
		4	6-10 per week
		5	11-30 per week
		6	More than 30 per week
34	Ever drank Cachaça (strong local alcohol)	1	No / occasionally
		2	No more than one glass per week
		3	2-5 per week
		4	6-10 per week
		5	11-30 per week
		6	More than 30 per week
35	Ever drank scotch, gin, vodka or other alcohol beverage	1	No / occasionally
		2	No more than one glass per week
		3	2-5 per week
		4	6-10 per week
		5	11-30 per week
		6	More than 30 per week

36	Number of years she has been drinking this amount	Num	
37	Number of years she drinks	Num	
38	Menarche	Num	
39A	Last menstrual period	Date	
39B	If not menstruated, reason	1	Post-partum
		2	Breast-feeding
		3	Menopause
40a	Type of menstrual absorbent: Sanitary pad	1	Yes
		2	No
40b	Type of menstrual absorbent: tampon	1	Yes
		2	No
40c	Type of menstrual absorbent: Cloth	1	Yes
		2	No
40d	Type of menstrual absorbent: Other type	1	Yes
		2	No
40d out	If other type (Q.40), mentioned	N-Num	
41	Felt Itching in genital area in the last 5 years	1	Never
		2	Sometimes (1-9 times)
		3	Many times (10 times and +)
42	Felt pain (burning) in the genital area in the last 5 years	1	Never
		2	Sometimes (1-9 times)
		3	Many times (10 times and +)
43	Vaginal discharge in the last 5 years	1	Never
		2	Sometimes (1-9 times)
		3	Many times (10 times and +)
44	Gynecologic products used	N-Num	
45	Homemade gynecologic products used	1	Yes
		2	No
45B	Type of homemade products	N-Num	
46	Discharge, itching or pain (burning) in the last 2 days	1	Yes
		2	No
47a	Use of vaginal shower douche.	1	Yes, always
		2	Yes, often
		3	Sometime
		4	Never
47b	Which product (for vaginal shower)	N-Num	
48	During menstruation, in addition to take a shower or a bath, does she wash genital organs	1	No
		2	Yes, once a day
		3	Yes, more than once a day
49	Ever had sores in the	1	Yes

	vaginal or vulva	2	No
50	Ever had venereal disease diagnosis	1	Gonorrhea
		2	Chancre
		3	Condylomas or venereal warts
		4	Syphilis
		5	Herpes
		6	Trichomonas
		7	Candidiasis
		8	Never
51	Ever had a prevention exam for cervical cancer, pap test or cytologic exam	1	Yes
		2	No
52	If yes (Q51), number of time	Num	
53	When was the last time (gynecologic exam)	1	Last year
		2	More than 1 year, less than 5
		3	More than 5
		8	Don't know
54	Age at the first sexual intercourse	Num	
55	Number of pregnancy	Num	
56	Number of normal delivery	Num	
57	Number of caesarian	Num	
58	Number of abortion	Num	
59	Year of the last pregnancy	Year	
60	This delivery (Q.59) was it a completed gestation	1	Yes
		2	No
61	Sexual relations during pregnancy	1	Yes
		2	No
62	Stop having sex after delivery	1	Yes
		2	No
63a	Age she starts to have sex at least once a week	Num	
63b	If never regular	1	
64	Number of lifetime sexual partners	Num	
65	Number of lifetime sexual partners (Q.64) that were regular for at least 6 months	Num	
66	Number of regular partners (Q.65) that were not loyal	Num	
67	Total number of sexual partner before the age of 20	Num	
68	Number of these partners	Num	

	(Q.67) that were less than 20 years old		
69	Number of these partners (Q.67) that were more than 30 years old	Num	
70	Total number of sexual partner after the age of 20	Num	
71	Number of these partners (Q.70) that were less than 20 years old	Num	
72	Number of these partners (Q.70) that were more than 30 years old	Num	
73	Lifetime number of years of interruption of the sexual relation (for more than one year)	Num	
74	Sexual intercourse frequency and duration	Num	
75	Sexual relation during menstruation	1	Always avoid it
		2	Sometimes
		3	Only on the first days
		4	Never
76	Genital organs washing BEFORE sexual relation	1	Always
		2	Sometimes
		3	Never
77	Genital organs washing AFTER sexual relation	1	Always
		2	Sometimes
		3	Never
78	Number of sexual partners in the last 5 years	Num	
79	Number of sexual partners in the last 5 years that were not loyal	Num	
80	Number of these partners (Q.78) that were less than 20 years old	Num	
81	Number of these partners (Q.78) that were more than 30 years old	Num	
82 S	Frequency of sexual relation during the last 5 years: BY WEEK	Num	

82 M	Frequency of sexual relation during the last 5 years: BY MONTH	Num	
82 A	Frequency of sexual relation during the last 5 years: BY YEAR	Num	
83	Number of sexual partners during the last 12 months	Num	
84	Number of sexual partners in the last 12 months that were not loyal	Num	
85	Number of these partners (Q.83) that were less than 20 years old	Num	
86	Number of these partners (Q.84) that were more than 30 years old	Num	
87 S	Frequency of sexual relation during the last12 months: BY WEEK	Num	
87 M	Frequency of sexual relation during the last 12 months: BY MONTH	Num	
87 A	Frequency of sexual relation during the last 12 months: BY YEAR	Num	
88	Contraception methods	1	Oral contraceptive
		2	Tubal ligation
		3	Vasectomy
		4	IUCD
		5	Condom
		6	Diaphragm
		7	Spermicide
		8	Withdrawal, rhythm (calendar), cervical mucus
		9	Other
		10	No method

88 B	Description of the "other" contraceptive method	N-Num	Example: Vaginal shower with vinegar, Vaginal shower with warm water, Anticontraceptive injection, Piece of Soap introduced in the vagina before relation, Japanese vaginal suppository, Pills of spermicide, Bandage of caustic liquid introduced in the vaginal, Vaginal spermicide suppository, Mint infusion in the vaginal or bean broth with salt, Wound dress in the cervix.
89	Age she starts contraceptive pills	Num	
90	Number of years taking contraceptive pills	Num	
91	During those years, resting period of contraceptive pills	1	Yes
		2	No
92	Number of years she stops contraceptive pills	Num	
93	How many years since tubal ligation	Num	
94	How many years since vasectomy of your partners	Num	
95	First time you use IUCD		
96	Still use IUCD	1	Yes
		2	No
97	Frequency of using condom	1	Rarely
		2	Sometimes
		3	Always
98	Frequency of using diaphragm	1	Rarely
		2	Sometimes
		3	Always
99	Spermicide use	1	Sole method
		2	Usually with diaphragm
		3	Usually with condom
100	Practice of anal penetration	1	Yes, often
		2	Yes, rarely
		3	No
101	Number of partners with anal penetration	Num	
102	Practice of anal penetration BEFORE vaginal penetration	1	Yes
		2	No
		3	Sometimes
103	Washing penis between anal and vaginal penetration	1	Yes
		2	No
		3	Sometimes

104	Changing condom between anal and vaginal penetration	1	Yes
		2	No
		3	Sometimes
		4	Did not use condom
105	Practice of oral sex	1	Yes, often
		2	Yes, rarely
		3	No
106	Number of partners with oral sex	Num	
107	Partner practices vaginal penetration before oral penetration	1	Yes
		2	No
		3	Sometimes

Note. Translated from Portuguese to English