Humoral response to HPV16 proteins in patients with anal high-grade squamous intraepithelial lesion and anal cancer

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Abstract

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Background: Considering the increasing incidence of human papillomavirus (HPV)-related anal cancer in the United States, a non-invasive screening strategy is warranted.

Objective: To assess potential association of anal HSIL or cancer with HPV16 antibody detection and estimate predictive ability of HPV16 antibody levels to determine HSIL or cancer status.

Design: This is a case-control study that included 67 cases of anal HSIL, 116 cancer cases, and 830 population-based matched controls for age and gender from the State of Washington. Sera were analyzed for HPV16 antibodies to L1 and early proteins (E1, E2, E4, E6, E7) by multiplex serology assay. Association between HSIL or cancer and HPV16 antibody seropositivity were examined using logistic

regression. Using classification tree (CART) and receiver operating characteristic analysis (ROC), we searched for HPV16 serological predictors of anal cancer.

Results: Invasive cancer was more common in women (63% of cases were women) and HSIL was more common in men (54% of cases were men). The median age at anal cancer diagnosis was 58 years in women and 54 years in men. Compared to men with HSIL, women with HSIL tended to be older (median age 50 versus 43 years). HPV16 L1 seropositivity was present in 77% of persons with anal cancer, 88% in those with HSIL, and 31% in controls. HPV16 antibodies to early proteins were more common in invasive cancer cases (E1 24%, E2 46.6%, E4 38.8%, E6 43.1%, E 37.9%) than in those with HSIL (E1 4.5%, E2 19.4%, E4 29.9%, E6 13.4%, E7 37.3%) and controls (E1 3.1%, E2 21.9%, E4 23.5%, E6 7.8%, E7 24.8%). L1 seropositivity was more strongly associated with HSIL (aOR 20.6; 95% CI, 9.7 – 37.3) than invasive cancer (aOR 8.8; 95% CI, 5.3 – 15.2). Seropositivity to HPV16 antibodies early proteins was significantly associated with invasive cancer: E1 aOR 19 (95% CI, 9.1 – 41.4), E2 aOR 3.7 (95% CI, 2.4 – 5.9), E4 aOR 2.7 (95% CI, 1.7 – 4.2), E6 aOR 8.7 (95% CI, 5.1 – 14.7), E7 aOR 1.8 (95% CI, 1.1 – 2.8). Only E7 was associated with increased risk of HSIL (aOR 1.8; 95% CI, 1.0 – 3.1). Higher E6 antibody levels (MFI >800) was associated with a 94% risk of anal cancer in this case-control study. Predictive models adding serological factors (L1, E1, and E6) along with baseline risk factors (age, smoking status, and number of sex partners) improved prediction of anal cancer (AUC 90%; 95% CI, 87% - 93%) compared with baseline risk factors alone (AUC 80%; 95% CI, 76% - 84%).

Conclusion: HPV16 seropositivity to early proteins was higher among persons with invasive anal cancer than persons with HSIL or controls. Incorporation of HPV antibodies to classification algorithms significantly improved the ability to predict anal cancer in this sample of cases and controls.

INTRODUCTION

Human papillomavirus (HPV) is a ubiquitous sexually transmitted virus that infects more than 90% of persons by adulthood; however, HPV-associated anal cancers develop in only a small proportion of infected persons.[1,2] Globally, 35,000 anal cancer cases per year in men and women are attributable to HPV, with higher frequency among women and HIV-positive MSM.[3] In the United States (U.S.), the incidence of anal cancer has increased by 2.2% each year over the past 10 years, with an estimated 8,500 cases and 1,100 deaths annually.[3] Although about a dozen HPV types have been declared human carcinogens, HPV16 is responsible for most HPV-driven cancers.[4] Persistent oncogenic HPV infection is associated with increased risk of anal high-grade squamous intraepithelial lesions (HSIL) and invasive cancer.[2]

The licensed HPV vaccine is effective in preventing anogenital HPV infections when provided prior to HPV acquisition. The vaccine is routinely recommended for men and women up to 26 years of age; however, most persons at risk of anal HSIL and cancer are older than the vaccine recommendation age limit and have already acquired HPV infection.[5] Furthermore, the impact of the vaccine has been reduced by the low immunization coverage rates in the U.S.[6] In the long-term, the preventative vaccine is an excellent tool for primary prevention and reduction of anal cancer incidence if rates of vaccination uptake improve.

Secondary prevention is needed to avert death and morbidity that impacts the quality of life among individuals who are already infected and developed HPV-associated disease. There are no national guidelines in the U.S. recommending secondary prevention of anal cancer, except for the State of New York.[7] At present, a large multicentric clinical trial in the U.S. (NCT02135419) is currently evaluating an approach to prevent anal cancer among HIV infected men and women. That study will compare screening using high-resolution anoscopy (HRA) and treatment of HSIL versus watchful waiting. HRA is an office-based procedure used to identify and treat anal HSIL through colposcopic examination

of the perianal area and anal canal with biopsy and ablation of any suspicious lesions.[8–10] HRA examination, which requires technical expertise by the clinician, is only available in a few centers across the U.S. and is associated with substantial discomfort for the patient. Non-invasive diagnostic methods to aid in predicting lesions that could progress to invasive cancer or that would lead to early detection of invasive cancer have not been established.

There are more than 200 types of HPV, approximately 12 HPV types are known as high risk due to their association with cancer.[11] High-risk HPV infects the basal layer of the stratified squamous epithelium and do not cause viremia.[12,13] Furthermore, the non-lytic replication and low protein expression early in the viral cycle result in evasion of immune recognition and delay of adaptive immune response.[12,13] Early (E1, E2, E4, E6, E7) and late (L1, L2) HPV genes are expressed during the virus life cycle.[12–14] Host measurable humoral response to HPV proteins differs by gender and anatomical sites of infection.[15] In a cohort study of female university students aged 18-20, antibody response to the L1 capsid was measured in 94% of women with prevalent HPV16 infection and 67% of students with incident HPV16 infection seroconverted within 24 months of viral acquisition.[16] A parallel study among male university students aged 18-21, showed that only 13% of men seroconverted within 24 months of HPV16 genital infection.[17]

The HPV oncoproteins E6 and E7 interact with p53 and pRb, proteins involved in cellular replication and DNA repair, and are therefore associated with the induction and maintenance of neoplastic transformation. Similar to the clinical use of viral oncoprotein antibodies in Merkel cell polyomavirus for skin cancer prognostication and surveillance[18], naturally-acquired antibodies to HPV oncoproteins have been examined as potential biomarkers to predict HPV-driven disease. For instance, two European case-control studies suggested that serum antibodies to HPV16 E6 might be a useful predictor of anal cancer.[19,20] However, results from a recent cohort study from the Netherlands did not support the use of HPV16 E6 seropositive as a marker of anal HSIL.[21]

Using a large sample from a population-based case-control study conducted in the State of Washington[22], we aimed to further assess the potential association of anal HSIL and invasive cancer with HPV16 antibodies detection. In addition, we evaluated the predictive ability of naturally acquired HPV antibody levels (L1, E1, E2, E4, E6, E7) to determine HSIL and invasive anal cancer status.

METHODS

Study Participants

The study population consists of a subset of participants previously recruited for a population-based case control study of HPV-driven anogenital cancers conducted at the Fred Hutchinson Cancer Research Center.[22] For this analysis, we included all cases with histologically confirmed diagnosis of anal disease - anal cancer or HSIL - and all controls from the prior study who had blood available for this analysis. Cases were residents of the Seattle area identified from the Cancer Surveillance System (CSS), a population-based tumor registry that serves the Seattle and Puget Sound areas in the State of Washington and participates in the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute. Cases were English-speaking women and men aged 18-74 years with anal HSIL or invasive anal cancer diagnosed between 1986 and 1998. HSIL or cancer diagnosis was confirmed by histopathologic review of tumors. The cancer registry ascertained 485 cases of anal HSIL and anal cancer, and 306 (63.1%) of those patients were interviewed for the original study. For the purpose of this work, we included 183 patients (60%) who completed the in-person interview and donated blood for serological evaluation.

Controls were selected from the same geographic area using random digit dialing, as described elsewhere.[23,24] Controls were frequency matched to cases based on age (5-year age distribution) and gender. All cases and controls resided in the three counties (King, Pierce, and Snohomish) that make up

the Seattle, Washington, metropolitan area. Of 2413 controls identified for the original study, 1700 agreed to be interviewed. A total of 1524 (89.1%) controls donated blood samples and a subset of those were selected for inclusion in this study (n=830). The original study and this sub-study were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board. For the parent case-control study, all participants provided written informed consent.

Data Collection

For the purpose of this work, we analyzed information already collected through questionnaires during the original study. After informed consent was obtained for the original study, both cases and controls were asked about demographic characteristics, sexual history, and smoking history. When answering questions, cases were asked to refer to a time prior to diagnosis. Cases provided signed informed consent for tumor block retrieval for HPV DNA testing. All participant's information gathered on questionnaires were entered into an electronic database.

Laboratory Methods

HPV antibody detection. Blood samples for HPV antibody testing were obtained for cases and controls and stored at -70°C until shipment to the German Cancer Research Center (Heidelberg, Germany). Sera were tested for HPV16 L1, E1, E2, E4, E6, and E7 proteins using a Luminex multiplex serology assay, as previously described.[25] Briefly, HPV antigens were affinity-purified and expressed in *E. coli* fusion proteins with N-terminal glutathione S-transferase. Antibodies bound to the antigens were detected via fluorescent reagents. To define a seropositive person, we used the Median Fluorescence Intensity (MFI) cutoffs predefined by the laboratory-specific cut-off values. The MFI values to define seropositivity were: L1,100; E1, 100; E2, 100; E4, 400; E6, 100; E7,100.

HPV DNA testing. Paraffin-embedded tissue blocks from 139 cases were tested for HPV DNA using polymerase chain reaction (PCR)-based methods as described previously.[26] For samples tested before

1996, PCR using L1 consensus primers (My09/My11)[27] were typed by Southern hybridization and oligonucleotides specific for HPV types 6/11, 16, 18/45, and 31.[28] For samples tested after 1996, we used restriction fragment analysis. In brief, HPV genotype was determined based on comparison of restriction patterns of amplified L1 fragments from cases tumor with patterns of HPV recombinant plasmids. When HPV genotype could not be determined based on restriction patterns, HPV genotype was assigned by automated sequencing of the L1 consensus products. To ensure that amplifiable DNA was present in the sample, biopsies with HPV DNA negative results were also tested by PCR to amplify 536- and 268-bp fragments of the beta-globin gene.

Statistical Analysis

The distribution of demographic characteristics by case-control status was investigated using frequencies. The case group was included either a) HSIL or b) invasive cancer; and these were compared to controls separately.

Univariable analyses exploring the associations between each of the HPV antibodies and anal HSIL or cancer as categorical outcome (HSIL yes/no) was performed. The relative risk of HSIL or cancer was estimated by calculating odds ratios (ORs). Multivariable logistic regression models were built by including potential confounders to evaluate changes in the magnitude of the relationship between HPV antibodies and outcomes (HSIL or invasive cancer). Potential confounders were selected a priori: age, gender, smoking status, and number of sex partners. Final models included all demographic and clinical variables regardless of their significance.

Multivariable classification and regression tree (CART) analysis was performed to evaluate if HPV16 antibody MFI detection patterns could distinguish anal HSIL or invasive cancer cases from controls. We aimed to identify mutually exclusive subgroups based on combinations of risk factors and specific HPV16 antibodies that are potentially associated with anal disease. In the CART analysis of anal

invasive cancer, the primary dependent variable was anal cancer and the independent variables were each of the HPV antibodies (L1, E1, E2, E4, E6, and E7) and risk factors (age, smoking status, and number of sex partners). The tree determines cutoffs optimally to minimize the variance within all subsequent branches. Cross-validated pruning was used to select trees with minimum overall deviance, avoiding over-fitting. Thus, the tree forecasts the risk of anal cancer by considering multiple HPV antibody detection and clinical factors at every node in the classification tree. Similar CART analysis was performed using anal HSIL as outcome.

Based on predictive variables established from the CART analysis, we built Receiver Operating Characteristic (ROC) curves and areas under the ROC curve (AUC) with associated 95% CI to evaluate the accuracy of predictors. Anal cancer was the binary outcome and relevant clinical and antibody levels were continuous predictors. For the baseline model ("risk factors"), we included biological (age) and behavioral factors (smoking and number of sex partners) that can affect the acquisition of HPV. In a stepwise approach, we introduced each of the HPV antibody variables that were determined to be predictive of anal cancer by the CART analysis (L1, E1, E6). Finally, we created models combining one or more antibody variables. Statistical analyses were performed in R (Version 3.4.3).

RESULTS

The study included 67 patients with anal HSIL, 116 patients with invasive anal cancer, and 776 matched controls. All study participants' sera were tested for antibodies to HPV16 L1, E1, E2, E4, E6 and E7. The demographic and clinical characteristics of cases and controls are presented in **Table 1**. Compared to men with HSIL, women with HSIL tended to be older (median age 50 versus 43 years). Similarly, among patients with invasive cancer, median age at diagnosis was older for women compared with men

(median age 58 versus 54 years). Invasive cancer was more common in women (63% of cases were women) and HSIL was more common in men (54% of cases were men). Compared to population-based controls, men and women with HSIL and invasive cancer were more likely to be current smokers and to have five or more sex partners. Among those with HSIL or invasive cancer, there were 139 available paraffin-embedded tissue blocks for HPV DNA detection; HPV16 was detected in 102 (73%) of tumor samples.

Association of antibody levels with HSIL and invasive cancer

HPV16 E1, E2, E4, and E6 seropositivity were more common in persons with invasive cancer than those with HSIL and controls. For example, the frequencies of antibody seropositivity varied from 24.1% for E1 to 54% for E2 among participants with anal cancer compared to 4.5% for E1 and 37.3% for E4 in HSIL cases and 3.1% for E1 and 23.5% for E4 in controls. A higher proportion of HSIL and invasive cancer cases than controls were seropositive for HPV16 L1 and E7 **(Table 2).** For instance, 88% of HSIL and 77% of cancer cases were seropositive to L1 protein compared to 31% of controls. To evaluate the effect of gender on antibody level and case-control status, we calculated deciles of response to each antibody assayed separately for cases and controls and examined the proportion of cases by gender. The proportion of cases among both men and women increased with higher antibody levels in a similar trend, suggesting that the relationship between antibody level and HSIL or cancer is similar by gender **(Figure 1).** Overall, MFI values of early antibodies were lower in males than women (data not shown).

We performed separate univariable analyses of factors associated with HSIL relative to controls and then of invasive cancer versus controls **(Table 2).** L1 seropositivity was more strongly associated with HSIL (OR 16.3, 95% CI 8.1 – 37.3) than invasive cancer (OR 7.6, 95% CI 4.9 – 12.3). However, seropositivity to each of HPV16 early antibodies was associated with invasive cancer: the odds ratio for E1 was 9.8 (95% CI 5.5 – 17.7), for E2 3.1 (95% CI 2.1 – 4.6), for E4 2.1 (95% CI 1.4 – 3.1), for E6 8.9 (95%

CI 5.7 – 14.0), and for E7 OR 1.9 (95% CI 1.2 – 2.8). In contrast, only E7 was statistically significantly elevated in persons with HSIL (OR 1.8, 95% CI 1.1 – 3.0).

On multivariable analysis, after adjusting for age, there remained significant associations between seropositivity to HPV antibodies and risk of invasive cancer. Associations between invasive cancer and E1 or L1 seropositivity were magnified following adjustment (E1 aOR 10.4, 95% CI 5.7 – 18.8 and L1 aOR 10.5, 95% CI 6.5 – 17.6). After adjusting for age and gender, the risk of cancer and E6 seropositivity was slightly attenuated (aOR 7.9, 95% CI 4.9 – 12.6). Interestingly, when the smoking variable was incorporated into the adjusted model, the odds ratio of invasive cancer for those with E1 seropositivity increased drastically (aOR 19.9, 95% CI 9.7 – 42.5). When the analysis was repeated adding number of sex partners to the model adjusted for age, sex, and smoking status, the variable had only a minor impact on the adjusted ORs. Results are summarized in **Table 2.**

Association of antibody levels with invasive cancer and HSIL by tree analysis

Classification and regression tree (CART) analyses were generated to predict HSIL or invasive cancer using risk factors previously shown to be associated with HPV-related infections (age, smoking status, and number of sex partners) and HPV16 antibodies L1, E1, E2, E4, E6, and E7 level on a continuous scale for MFI units. Comparing cases with invasive cancer to controls, the CART analysis selected five measures associated with cancer **(Figure 2):** higher MFI for three antibodies, E6, L1, and E2, being a current smoker, and being older age. The analysis predicted that the lowest risk group consisted of patients with low E6 and low L1 antibody levels, who accounted for only 5% of invasive cancer diagnoses. Interestingly, persons at highest risk of cancer could be simply described using only one predictor: higher E6 antibody levels (MFI $\geq$~800), associated with 94% risk in this case-control study. The next highest risk group was composed of non or former smokers with elevated L1 (MFI $\geq$~425) and mildly elevated E6 (MFI >~ 182) whose risk of invasive cancer was 80%. In older smokers with low E6

levels (MFI ≤~800), but high L1 (MFI ≥~425) and E2 antibody levels (MFI ≥~8), the risk of invasive cancer was 73%. When building a similar tree for anal HSIL, the strongest predictor was the level of L1 antibodies **(Figure 3)**. HSIL risk was highest (51%) in those ever smokers with high levels of L1 antibody (MFI ≥~900) and lowest in persons with low L1 antibody levels (4%). Factors such as early HPV antibodies, age, and number of sex partners were not predictive in this analysis.

Ability of HPV antibody levels to predict cancer

From the CART analysis, we identified several risk factors for anal invasive cancer. To improve population-level risk assessment, we analyzed the sensitivity and specificity of these risk factors based on generating ROC for risk factors alone and in combination with HPV antibody levels. The predictive ability measured by area under the ROC (AUC) containing only the risk factors age, smoking status, and number of sex partners resulted in an AUC of 0.80 (95% CI 0.76 – 0.84) **(Figure 4)**. When compared to the model with risk factors only, the model including antibody levels in addition to risk factors yielded a higher predictability of anal cancer (e.g., AUC of 0.87 with 95% CI 0.83-0.90 if adding L1 and AUC of 0.85 if adding E6, 95% CI 0.82-0.89). Also, the models comprised of a higher number of antibody measurements had higher predictive power for anal cancer.

If risk factors and antibody levels could be used to identify candidates to be targeted for biopsy assessment of cancer, we could assess the sensitivity/specificity tradeoff based on the highest AUC (model with clinical factors and L1, E1, and E6). If we set a sensitivity target of 90% to ensure identifying 90% of cases, the thresholds for the best of these tests would only provide a specificity of about 65%. Assuming a cancer prevalence of 2%, the positive predictive value (PPV) for identifying an invasive anal cancer would be 5.0% and the negative predictive value (NPV) would be 99.7%. In a population with a higher prevalence of 12%, the PPV increases to 26.0% and NPV would be 97.9%. If we set a sensitivity target of 80%, the specificity would be about 81%. The PPV for identifying an invasive anal cancer would

be 7.9% if the disease prevalence is 2% and 36.5% if the cancer prevalence is 12%. The NPV of the AUC model would be 99.5% and 96.7%, respectively **(Table 3)**.

DISCUSSION

This large population-based case-control study provides important insights into HVP16 antibody detection and risk of anal HSIL or invasive cancer. We determined a statistically significant association between HPV16 antibodies seropositivity to L1 capsid and all early proteins and invasive anal cancer. An increased risk of anal HSIL was also found in persons seropositive for L1 and E7, when compared with controls. Based on a predictive analysis that included baseline risk factors and quantitative HPV antibody measures, our results indicated that an elevated E6 (using cutoff MFI $\geq$ 800) was associated with the highest risk of invasive cancer. In contrast, anal HSIL was best predicted by high L1 levels, best predictor of invasive cancer. The discriminatory power of HPV antibodies as potential biomarker of anal cancer, assessed by ROC analysis, suggested that HPV serology could aid clinical management, but the utility of this tool may be limited by the low disease prevalence in the general population.

The detection of antibodies to early proteins reflect virus-driven epithelial cell division in active infection.[12,13] Viral integration into the host genome leads to a decreased expression of E1, E2, and E4.[14] By contrast, E6 and E7 oncoproteins are upregulated and their expression is maintained during the neoplastic state.[14] Several studies have explored the potential use of HPV antibodies as biomarkers of anal cancer.[19–21] A prospective study in Europe by Kreimer et al. found that 30% of patients with anal cancer were E6 seropositive compared with only 0.6% of controls.[19] The increased risk of anal cancer was highly associated with E6 antibody seropositivity (OR 75.9, 95% CI 17.9 – 312) and E6 antibody was detected a mean of 8 years before the diagnosis of anal cancer. Seropositivity to L1, E1, and E7 was also significantly associated with increased risk of anal cancer in that study. Another study by Bertish et al.

among HIV-positive patients (n=41 cases with anal cancer and n=114 controls) showed that the proportion of anal cancer cases seropositive to L1 and E6 (76% and 22%, respectively) were higher compared with controls (39% and 0%, respectively). Furthermore, increased risk of anal cancer was found in seropositive patients for L1 (OR 4.25,95% CI 2.0 – 10.2) or E6 (OR ∞, 95% CI 1.08 – 1.42).[20] Using the same laboratory methods but with lower seropositivity cutoffs, we also observed strong associations between invasive cancer and antibodies to HPV16 capsid and early proteins when compared with controls.

Anal HSIL is presumed to lead to invasive cancer.[29] Identification and treatment of HSIL before progression to cancer could be an effective strategy to reduce the incidence of anal cancer. High resolution anoscopy with biopsy of suspicious lesions has become the gold standard tool for detection of HSIL.[30] Alternative non-invasive screening tools have been evaluated, but findings across the literature remain inconsistent. Several studies have shown heterogenous results when evaluations included demographic, behavioral, and clinical characteristics for prediction of HSIL.[2,31–36] Viral factors screening approach (e.g., high-risk HPV typing, anal cytology, HPV DNA methylation, and HPVE6/E7 mRNA in anal swabs) lack sensitivity or specificity.[37–39] HPV antibodies as predictor of HSIL has been recently evaluated in a longitudinal study conducted by Marra et al. in the Netherlands (n=50 persons with anal HSIL).[21] This study did not support the association between HSIL and HPV antibodies detection; antibodies examined included L1, E1, E2, E6, and E7. In our study, we observed an increase in the likelihood of HSIL in seropositive patients for L1 or E7. Although we used the same serological assay, Marra et al. utilized a more stringent serological cutoff to determine seropositivity for HPV antibody.

Antibodies to HPV early proteins have also been evaluated as potential biomarkers of HPV-driven cancer across genital and oropharyngeal sites. For example, 50% of women with cervical cancer will develop E6 antibody response compared with 3-9% of control women.[40] In a European case-control study of women with cervical HSIL (n=425), invasive cancer (n=184), and cancer-free controls (n=1,218),

HPV16 E6 antibody was detected in 11% of women with invasive cancer compared with 1.6 % of women with HSIL and 1.4% of control women.[41] Several studies have also demonstrated high E6 seroprevalence in patients with HPV-driven oropharyngeal cancer. For instance, in a study of 115 patients with HPV-oropharyngeal cancer, 98 (85%) persons were seropositive to HPV E6 at diagnosis.[42] In patients with E6 seropositive at diagnosis, the mean antibody level significantly declined at 2 years post-treatment. Antibodies to HPV proteins were also found to be strong predictors of future oropharyngeal cancer. Kreimer et al. showed that 42.3% of 52 patients with oropharyngeal cancer and 0.5% of 924 control subjects were HPV16 E6 seropositive and antibody levels remained stable for up to 13 years prior to cancer diagnosis.[43]

Unlike for most other infections, even virologically documented infection with HPV does not universally result in detectable antibodies. For example, in a cohort of 1,595 patients attending a sexually transmitted disease clinic in the U.S., only 30.2% of women and 18.7% of men had detectable antibodies to HPV.[44] In serosurveys in general population, approximately 40.5% of women and 19.4% of men have L1 antibodies to any 9 HPV vaccine types.[45] This limits the usefulness of seroprevalence studies to assess the frequency of HPV infection. Studies consistently have shown a stronger immune response in women compared to men. The mechanisms explaining this difference are not well understood. One potential explanation for seroprevalence differences is that the immune response of the keratinized genital epithelium in men is lower compared to women's mucosal immune system.[17,46] Another hypothesis is that HPV infections in men tend to be shorter than women.[47] The use of specific antibodies to HPV oncoproteins is promising as seroconversion in persons without an underlying HPV disease would not be expected. Several studies evaluating the potential utility of antibodies against HPV oncoproteins showed higher seropositivity among patients with HPV-driven cancer when compared to controls.[43,48,49] Although not all cases seroconvert, only a small proportion of controls are seropositive. In a pooled analysis of controls (n=4666) without diagnosis of HPV-driven cancer, only 32 (0.7%) individuals

had E6 seropositivity.[50] In our study, the proportion of HPV16 E6 seropositivity was significantly higher among patients with invasive cancer (43.1%) compared with patients with HSIL (13.4%) or controls (7.8%). The positivity rate among controls could be explained by potential laboratory error or subclinical HPV-driven dysplastic process.

Cancer surveillance programs are intended to detect premalignant disease or prevalent cancer at an early stage. Cervical cancer screening, one of the most successful cancer prevention programs implemented, intends to detect and treat cervical HSIL before progression to cancer. When an abnormal cervical cytology is determined, women are referred for colposcopic exam and for direct visualization of lesions under magnification. If cervical lesions are found, biopsy are obtained and lesions consistent with HSIL are treated. Given biological similarities between cervical and anal cancer, secondary prevention strategies used for cervical cancer have been adopted for the evaluation of anal cancer, such as anal cytology (adaptation of Papanicolaou) and high-resolution anoscopy (modification of cervical colposcopy).[9] Unlike the cervix, anal Pap cytology is less sensitive for identification of HSIL and there is not a standardized management algorithm for abnormal results.[38] Furthermore, performing HRA can be subjective, technically challenging, and require a long learning curve. Therefore, guidelines have been published proposing a minimum competency for the clinical practice of HRA (minimum of 50 HRAs per year and identification >20 cases of HSIL, <5% of unsatisfactory anal cytology sample, procedure duration of less than 15 minutes, cytological and histological HSIL concordance of >90%).[8] Contrasting with the quantity of cervical colposcopy clinicians in the U.S., the number of HRA provider and clinical centers are fewer. Given the multiple limitations of the universal implementation of HRA for HPV-disease screening, non-invasive screening tools are needed for detection of anal HSIL and early invasive anal cancer. Identification of antibodies, or antibody combinations, that are associated with the presence of early anal neoplasia could facilitate disease detection at early stages, when treatment is less invasive and more effective.

Since a single diagnostic and prognostic marker of anal HPV disease has not been recognized, we aimed to identify clinical and laboratory predictors that can guide providers in making diagnosis and treatment decisions. Predictor tools can help characterize patients that are at higher risk of invasive cancer to offer closer surveillance for HSIL progression to malignancy. Notably, the results of our regression tree analysis suggested that increased HPV16 antibody response to E6 protein appears as a powerful anal cancer predictor (94% risk in our case-control sample with 12% invasive anal cancer prevalence). Elevated L1 antibody levels and current tobacco use were also predictive of cancer in this study (80% risk). On the other hand, 5% risk of invasive cancer was determined in those with lower levels of E6 (MFI <800) and L1 (MFI <425) antibodies. When exploring predictors of HSIL, antibodies to early proteins were not helpful in assessing risk of disease. The L1 antibodies and smoking status were only 50% predictive of disease. After identifying HPV antibodies associated with anal cancer by classification tree analysis, we developed models for predicting anal cancer by estimating the AUC of the ROC. The antibody (L1, E1, E6) based risk model improved anal cancer prediction (AUC 0.90, 95% CI 0.87 – 0.93). compared with risk factor model (AUC 0.80, 95% CI 0.76 – 0.84). Moreover, the antibody predictor model had the potential to achieve high sensitivity for identifying cancer and acceptable specificity for the defined sensitivity. Considering a sensitivity of 80% which in turn provided a specificity of 81%; our model indicated a positive predictive value of 36.5% and misdiagnosis of 3.3% of persons with a negative result, when the pretest probability was set at 12%. However, the anticipated probability in general population is 1 in 500 during a lifetime. Hence, the clinical application of this predictive tool may be limited by disease prevalence. Furthermore, the high negative predictive value of the predictive tool suggests that HPV antibody testing might help identify patients that can be followed conservatively after an HPV16 antibody result.

A limitation of this study is lack of examination to exclude HPV-associated lesions and cancer in controls and the resulting potential misclassification of HSIL and cancer status. Although

misclassification of cancer among controls can occur, this is unlikely as anal cancer is rare. This potential misclassification would result in an attenuation of the true association between the antibodies and risk of disease. Additionally, this analysis is limited by the study design, including unmeasured confounders not addressed in the analysis such as HIV status. At the time data were collected, HIV testing was infrequent. However, multiple studies have shown that HIV-infected individuals have an increased risk of HPV-associated anal lesions. Because HIV status was not assessed during data collection, it is not known whether the study accurately sampled the populations that largely represent the burden of disease today, limiting the generalizability of this study. The study is also limited due to the unavailability of HPV tests on all tumor tissue samples, which could affect the precision of the study estimates. Lastly, the participation rate for in-person interview in the original study was 63.1% among cases and 68% among controls. Despite these limitations, this is one of the largest case-control studies of HSIL and anal cancer to date.

CONCLUSION

Non-invasive screening tools are needed for detection, prognosis, and surveillance of anal HSIL and early invasive anal cancer. In our study, patients with anal HSIL or invasive anal cancer had a distinct immune response for multiple HPV antibodies. Our study strengthens the evidence that E6 may predict cancer progression among persons with HSIL. Further, we demonstrated that HPV serology (L1, E1, E6) can identify persons with invasive cancer with high sensitivity and specificity. Thus, HPV serology has the potential to improve anal cancer prediction. Future research should focus on replication of our finding in other populations at high risk of anal cancer.

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Table 1. Demographic and clinical characteristics of participants in included in the study.

	HSIL (N= 67)		**Invasive cancer** (N= 116)		**Controls** (N= 830)	
	Women N= 31 (46%)	**Men** N= 36 (54%)	**Women** N= 73 (63%)	**Men** N= 43 (37%)	**Women** N= 670 (81%)	**Men** N= 160 (19%)
Age (year)						
Median (range)	50 (20, 69)	43 (23, 71)	58 (29, 74)	54 (32, 73)	46 (18, 78)	50 (20, 74)
Smoking status						
Never	5(16%)	5 (14%)	20 (27%)	8 (19%)	359 (54%)	65 (41%)
Former	7(23%)	9 (25%)	14 (19%)	9 (21%)	163 (24%)	58 (36%)
Current	19 (61%)	22 (61%)	39 (53%)	26 (60%)	148 (22%)	37 (23%)
Missing	0	0	0	0	0	0
Number of sex partners						
1	1 (3%)	6 (17%)	8 (11%)	4 (9%)	209 (31%)	40 (25%)
2-4	16 (52%)	8 (22%)	21 (29%)	10 (23%)	202 (30%)	38 (24%)
≥5	14 (45%)	22 (61%)	43 (59%)	29 (67%)	253 (38%)	79 (49%)
Missing	0	0	1	0	6	3
Calendar period at diagnosis						
1978-1989	3 (10%)	2 (5%)	8 (11%)	7 (16%)	53 (8%)	22 (14%)
1990-1994	16 (52%)	15 (42%)	33 (45%)	12 (28%)	267 (40%)	51 (32%)
1995-1998	12 (38%)	19 (53%)	32 (44%)	24 (56%)	350 (52%)	87 (54%)

Abbreviations: HSIL, high-grade squamous intraepithelial lesion.

Table 2. Association of detection of HPV16 antibodies and anal disease.

Ab type	**Controls** N (%)	**HSIL** N (%)	**OR[a] [95%CI]**	**aOR[b] [95%CI]**	**Cancer** N (%)	**OR[c] [95%CI]**	**aOR[d] [95%CI]**	**aOR[e] [95%CI]**	**aOR[f] [95%CI]**	**aOR[g] [95%CI]**
E1	26 (3.1)	3 (4.5)	1.4 (0.3, 4.3)	2.5 (0.5, 8.8)	28 (24.1)	**9.8 (5.5, 17.6)**	**10.4 (5.7, 18.8)**	**10.5 (5.6, 19.9)**	**19.9 (9.7, 42.5)**	**19.0 (9.1, 41.4)**
E2	182 (21.9)	13 (19.4)	0.9 (0.4, 1.6)	1.0 (0.5, 2.0)	54 (46.6)	**3.1 (2.1, 4.6)**	**3.5 (2.3, 5.2)**	**3.5 (2.3, 5.3)**	**3.6 (2.3, 5.7)**	**3.7 (2.4, 5.9)**
E4	195 (23.5)	20 (29.9)	1.4 (0.8, 2.4)	1.7 (0.9, 3.1)	45 (38.8)	**2.1 (1.4, 3.1)**	**2.2 (1.5, 3.4)**	**2.3 (1.5, 3.6)**	**2.6 (1.6, 4.1)**	**2.7 (1.7, 4.2)**
E6	65 (7.8)	9 (13.4)	1.8 (0.8, 3.7)	1.5 (0.6, 3.2)	50 (43.1)	**8.9 (5.7, 14.0)**	**8.6 (5.5, 13.5)**	**7.9 (4.9, 12.6)**	**8.4 (5.1, 13.9)**	**8.7 (5.1, 14.7)**
E7	206 (24.8)	25 (37.3)	**1.8 (1.1, 3.0)**	**1.8 (1.0, 3.1)**	44 (37.9)	**1.9 (1.2, 2.8)**	**1.8 (1.2, 2.7)**	**1.6 (1.1, 2.5)**	**1.8 (1.1, 2.7)**	**1.8 (1.1, 2.8)**
L1	259 (31.2)	59 (88.1)	**16.3 (8.1, 37.3)**	**20.6 (9.7, 49.7)**	90 (77.9)	**7.6 (4.9, 12.3)**	**10.5 (6.5, 17.6)**	**11.5 (7.0, 19.5)**	**10.6 (6.4, 18.1)**	**8.8 (5.3, 15.2)**

a. Unadjusted OR was estimated comparing cases of HSIL and all controls.
b. Adjusted OR for age, gender, smoking status, and number of sex partners.
c. Unadjusted OR was estimated comparing cases of invasive cancer and all controls.
d. Adjusted OR for age.
e. Adjusted OR for age and gender.
f. Adjusted OR for age, gender, and smoking status.
g. Adjusted OR for age, gender, smoking status, and number of sex partners. This was the final model.

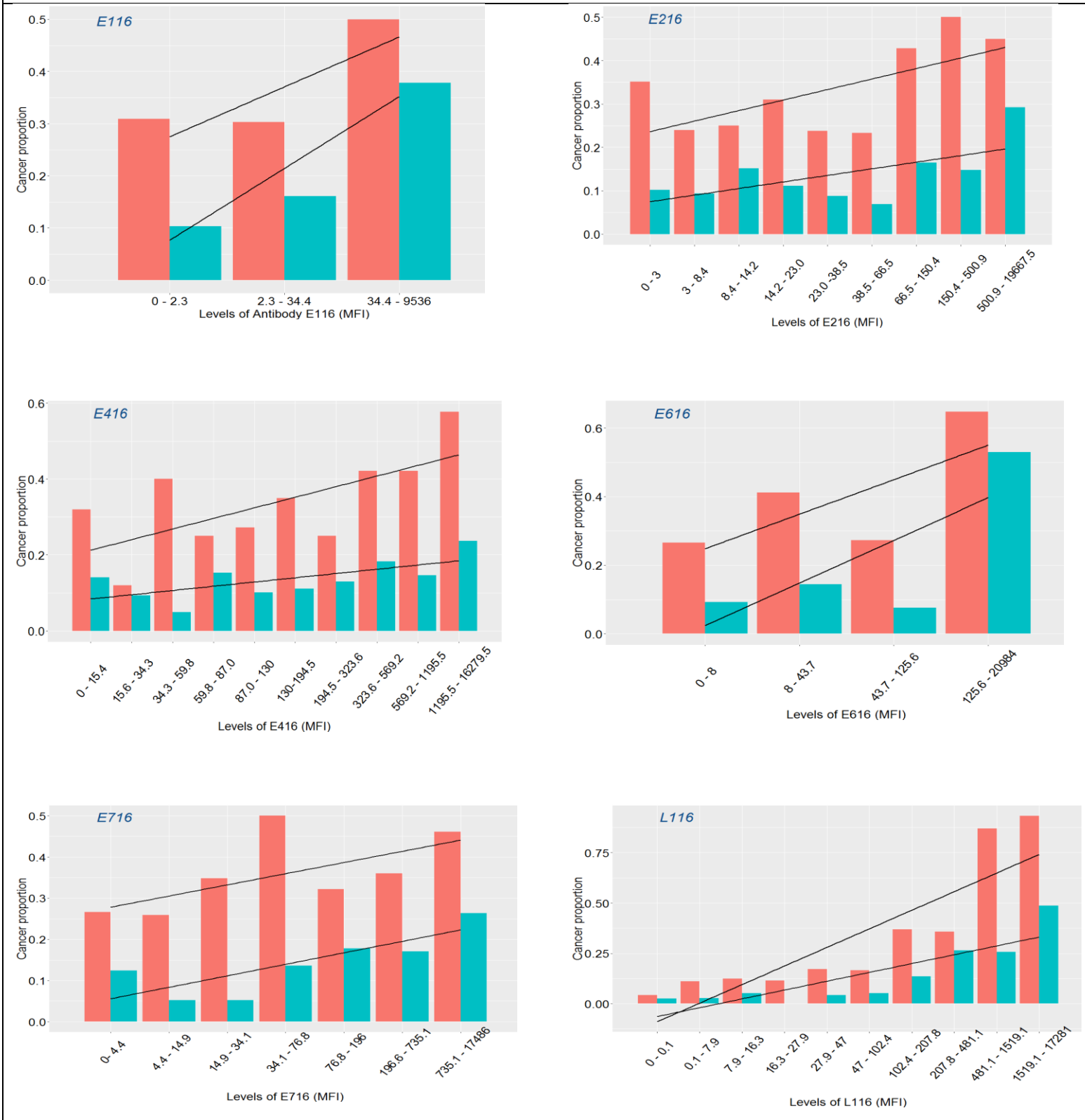
Abbreviations: Ab, antibody; aOR, adjusted odds ratio; OR, odds ratio; n, sample size; HSIL, high-grade squamous intraepithelial lesion.

Table 3. Predictive values for clinical and serological factors for different prevalences.

Sensitivity	Specificity	Prevalence	PPV	NPV
0.9	0.65	2%	5.0%	99.7%
0.9	0.65	4%	9.7%	99.4%
0.9	0.65	6%	14.1%	99.0%
0.9	0.65	8%	18.3%	98.7%
0.9	0.65	10%	22.2%	98.3%
0.9	0.65	12%	26.0%	97.9%
0.8	0.81	2%	7.9%	99.5%
0.8	0.81	4%	14.9%	99.0%
0.8	0.81	6%	21.2%	98.4%
0.8	0.81	8%	26.8%	97.9%
0.8	0.81	10%	31.9%	97.3%
0.8	0.81	12%	36.5%	96.7%

This table presents the sensitivity/specificity tradeoff based on the best ROC curve model (clinical factors and HPV16 L1, E1, and E6) among different anal cancer prevalences.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

Figure 1. Proportion of anal cancer cases by specific HPV16 antibody level for each gender. Male Female



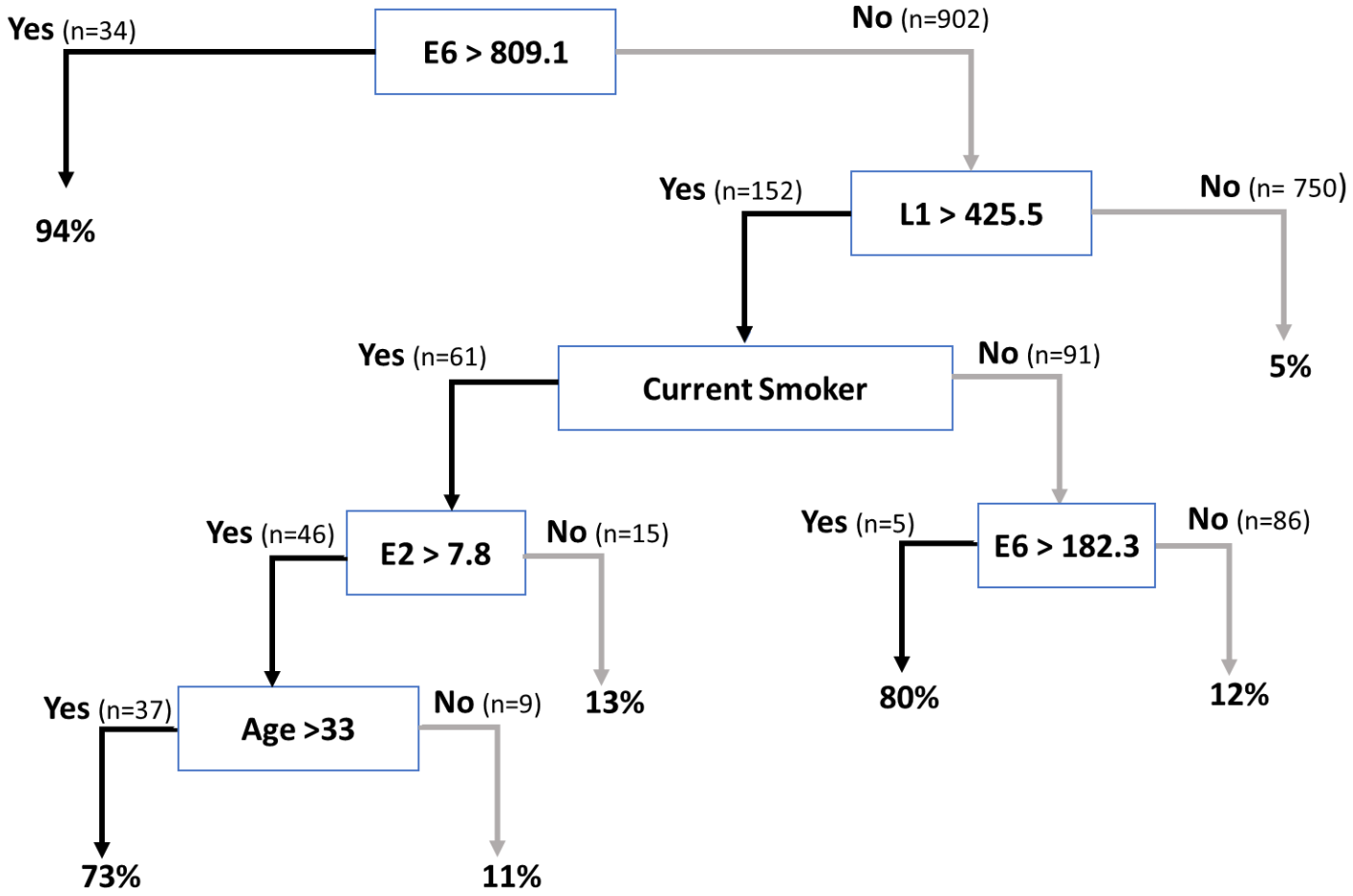


Figure 2. Classification and regression tree results for risk of invasive anal cancer, assessing its association with HPV16 antibody to L1, E1, E2, E4, E6, and E7, age, smoking status, and number of sex partners. The tree analysis selected predictors associated with anal cancer (E6, L1, E2, smoking status, and age). The risk of invasive anal cancer is indicated at the bottom of each branch.

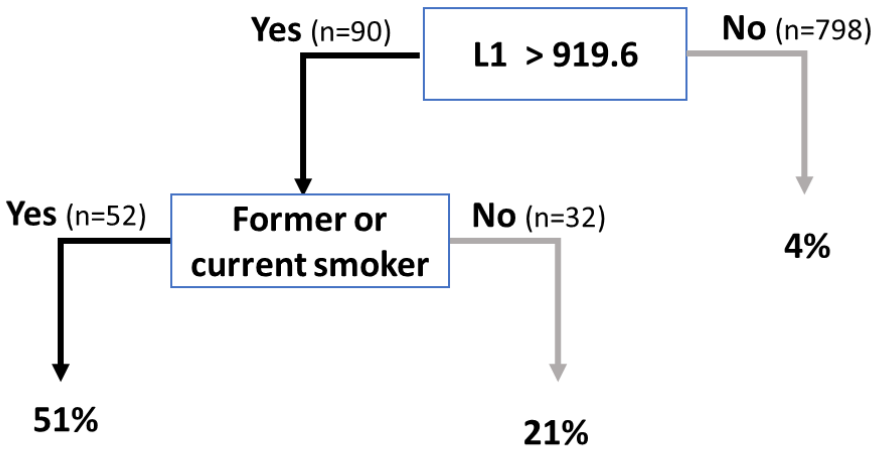


Figure 3. Classification and regression tree results for risk of anal HSIL, assessing its association with HPV16 antibody to L1, E1, E2, E4, E6, and E7, age, smoking status, and number of sex partners. The tree analysis selected predictors associated with HSIL (L1 and smoking status). The risk of anal HSIL is indicated at the bottom of each branch.

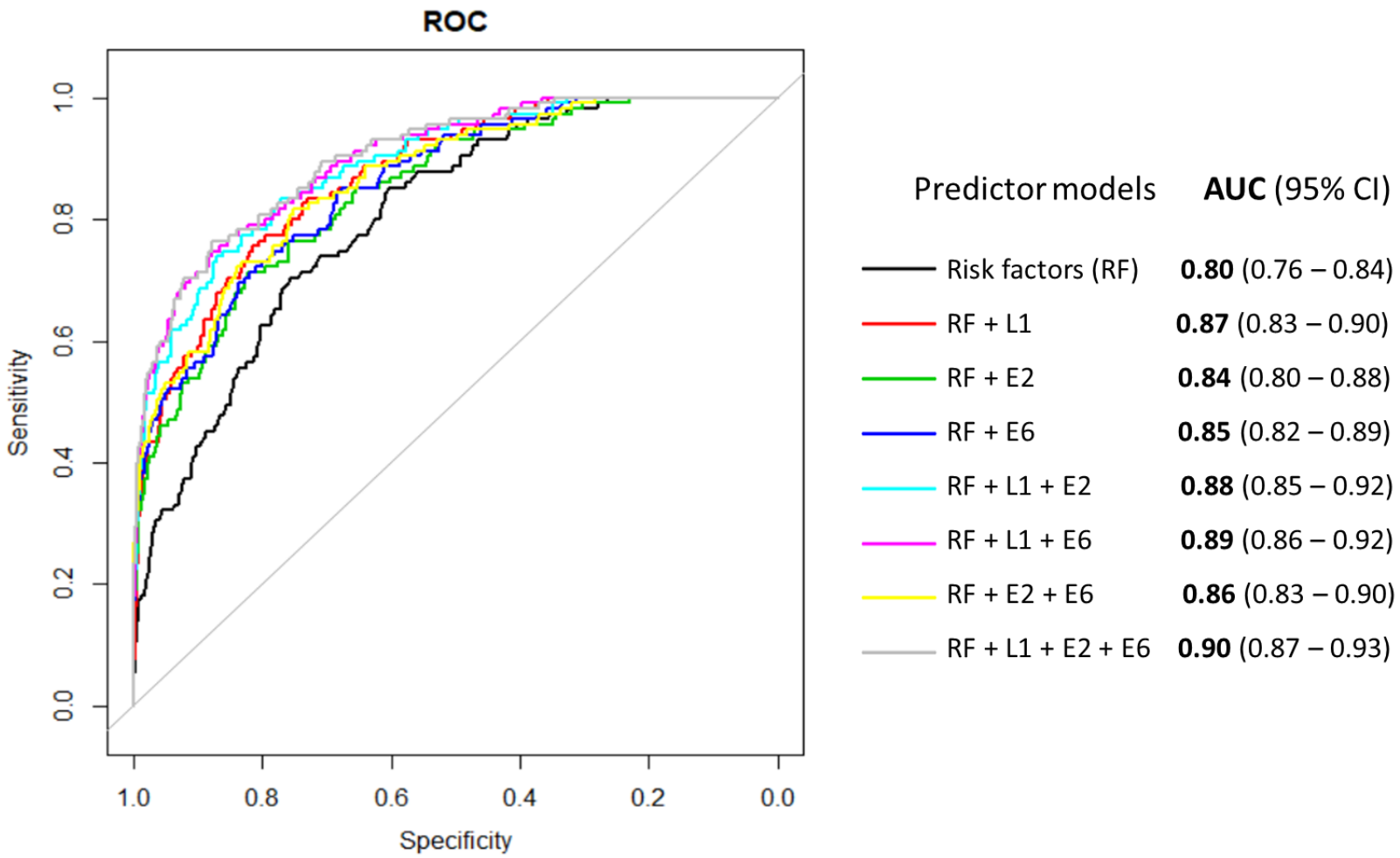


Figure 4. Receiver operating characteristic curves of the models containing risk factors (smoking, age, number of sex partners) and HPV16 antibody types. HPV16 antibody types were selected based on predictors identified by tree analysis.