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Human papillomavirus genotyping, human papillomavirus mRNA expression, and p16/Ki-67 cytology to detect anal cancer precursors in HIV-infected MSM

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Abstract

Objective—Anal cancer incidence is high in HIV-infected MSM. Screening for anal intraepithelial lesions and cancers is performed at specialized clinics and relies on high-resolution anoscopy (HRA) and anal cytology. Both approaches have limited reproducibility and sensitivity for detecting anal cancer precursors. We evaluated biomarkers for human papillomavirus (HPV)-related disease in a population of HIV-infected MSM.

Methods—A cross-sectional screening study with passive follow-up included 363 MSM followed at a HIV/AIDS clinic. All men had anal cytology samples taken and were evaluated using HRA and anal biopsies. Using a composite endpoint of biopsy results and cytology, we compared the performance of HPV16/18 genotyping, HPVE6/E7 mRNA expression, and p16/Ki-67 cytology to detect high-grade anal intraepithelial neoplasias (AINs).

Results—For all biomarkers analyzed, there was a significant trend of increasing percentage of men testing positive with increasing severity of disease ($P < 0.001$). HPV DNA testing had the highest sensitivity for anal intraepithelial neoplasia grade 2 and anal intraepithelial neoplasia grade 3 (AIN3), followed by p16/Ki-67, HPVE6/E7 mRNA testing, and HPV16/18 genotyping. The highest Youden's index was observed for HPVE6/E7 mRNA testing, followed by HPV16/18 genotyping, p16/Ki-67 cytology, and HPV DNA testing. Increasing the threshold for positivity of

p16/Ki-67 to five or more positive cells led to significantly higher specificity, but unchanged sensitivity for detecting AIN3.

Conclusion—Molecular features of anal disease categories are similar to those of corresponding cervical lesions. Biomarkers evaluated for cervical cancer screening may be used for primary anal cancer screening or to decide who should require immediate treatment vs. expectant management.

Keywords

anal cancer screening; HIV; human papillomavirus; human papillomavirus mRNA; MSM; p16

Introduction

Anal cancer is uncommon in the general population, with annual incidence rates of about 2/100 000 in the United States. However, the risk of anal cancer is substantially increased in certain populations, such as MSM, and human immunodeficiency virus (HIV)-infected men and women. In MSM, anal cancer rates are estimated to be 40/100 000. The risk of anal cancer may be two-fold to four-fold higher in HIV-infected MSM than in HIV-uninfected MSM [1]. An increase in anal cancer annual rates among HIV-infected men has been reported after introduction of HAART [2].

It is widely recognized that most anal cancers and anal cancer precursor lesions [anal intraepithelial neoplasia grade 3 (AIN3)] are caused by persistent infections with carcinogenic human papillomaviruses (HPVs), analogous to cervical cancers and cervical precancers [3,4]. Cervical cancers develop over decades from HPV infection in the squamo-columnar transition zone, through viral persistence, progression to precancer, and invasion [5]. The possibility to detect and treat cancer precursors and the long time before precancer becomes invasive has made cervical cancer screening programs possible. Where effective cervical cancer screening programs have been implemented, substantial reductions in cervical cancer incidence have been observed [6].

Thus, screening for anal cancer in high-risk populations such as HIV-infected MSM may lead to effective secondary anal cancer prevention. In fact, anal cancer screening among high-risk populations is already practiced at specialized centers. Screening, currently, strongly relies on high-resolution anoscopy (HRA), a direct visual method that has limited sensitivity for detecting anal precancer [7,8]. Anal cytology is increasingly used for screening of high-risk populations, but may be hampered by limited sensitivity similar to cervical cytology [9].

Although much less studied, it is conceivable that the key steps in anal carcinogenesis are similar to those that are well defined in the cervix. Most cervical HPV infections clear after a short period or at least become undetectable; a small subset persists and may develop from a transient to a transforming infection that is characterized by increased expression of HPV oncogenes. Therefore, several promising biomarkers that identify transforming infections and precancer of the cervix, such as HPVE6/E7 mRNA detection and p16/Ki-67 staining [10], might be similarly useful for clinically important anal infections. Also, in the cervix, HPV16 and HPV18 are the most carcinogenic types; infections with HPV16 are associated

with a high enough immediate risk of cervical intraepithelial neoplasia grade 3 (CIN3) that referral to colposcopy may be warranted [11]. There is some evidence that HPV16 may cause an even greater proportion of anal cancers than it does cervical cancers [12].

Here, we evaluated several biomarkers including HPV DNA testing, HPV DNA genotyping for HPV16 and HPV18, HPVE6/E7 mRNA testing, and p16/Ki-67 staining in a population of HIV-infected MSM enrolled at an anal cancer screening clinic in the Kaiser Permanente Northern California Health Maintenance Organization.

Methods

Study population

The study was based at the San Francisco Kaiser Permanente Northern California (KPNC) Anal Cancer Screening Clinic. We enrolled men who were identified as HIV-infected through the Kaiser HIV registry, who were 18 years or older, who were not diagnosed with anal cancer prior to enrollment, and who provided informed consent. In total, 363 men were enrolled between August 2009 and June 2010. The study was reviewed and approved by the institutional review boards at KPNC and at the National Cancer Institute. All participants were asked to complete a self-administered questionnaire to collect risk factor information. Additional information on HIV status and medication, sexually transmitted diseases, and histopathology results were abstracted from the KPNC clinical database. For 86 of the 271 patients who had no anal intraepithelial neoplasia grade 2 (AIN2) or AIN3 identified at the enrollment visit, follow-up information from additional clinic visits up to December 2011 was available and included in the analysis to ensure good ascertainment of prevalent disease, as anoscopy has less-than-perfect sensitivity.

Cytology, anoscopy, and histology

During the clinical examination, two cytology specimens were collected by inserting a wetted swab into the anal canal up to the distal rectal vault and withdrawing with rotation and lateral pressure. Both specimens were transferred to PreservCyt medium (Hologic, Bedford, Massachusetts, USA). A third specimen was collected for routine *Chlamydia trachomatis* and *Neisseria gonorrhea* testing. After specimen collection, participants received a digital rectal examination followed by HRA. All suspicious-appearing lesions identified in HRA were biopsied and sent for routine histopathological review by pathologists at the KPNC. From the first specimen, a ThinPrep slide (Hologic, Bedford, Massachusetts, USA) was prepared for routine Pap staining and reviewed by two cytopathologists (T.M.D. and D.T.). Cytology results were reported analogous to the Bethesda classification for cervical cytology [13], using the categories negative for intraepithelial lesion or malignancy (NILM), atypical squamous cell of undetermined significance (ASC-US), atypical squamous cells – cannot exclude high-grade lesion (ASC-H), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesions AIN2 (HSIL-AIN2), and HSIL-AIN3. We observed moderate agreement between two independent expert cytology reviews [14]. In this analysis, we report the primary cytology result from KPNC pathology. Histology results were reported as negative, condyloma acuminata, and AIN grades 1–3.

Human papillomavirus DNA testing

Both HPV DNA tests, Cobas 4800 and Linear Array, were conducted from the second container by Roche (Pleasanton, California, USA) blinded to all study data. To prepare DNA for both linear array and Cobas, automated sample extraction was performed as follows: 500 µl of the PreservCyt specimen was pipetted into a secondary tube (Falcon 5-ml polypropylene round-bottom tube, 12-by-75-mm style, nonpyrogenic, sterile). The tube was capped, vortexed, uncapped, placed on the x-480 specimen rack and loaded onto the x-480 sample extraction module of the Cobas 4800 system. The x-480 extraction module then inputs 400 µl of this material into the specimen preparation process. Testing for Cobas 4800 and Linear Array was performed as previously described [15]. Both tests use amplification of the globin gene as control for presence of sufficient DNA. Four samples without globin amplification were considered invalid and excluded from the analysis.

RNA proofer human papillomavirus mRNA testing

HPV mRNA testing was conducted from the second container by NorChip (Klokkarstua, Norway) blinded to all study data. DNA/RNA was isolated from 5-ml PreservCyt specimens by using the NucliSENS easyMAG system (bioMerieux, Marcy l'Etoile, France). The mRNA testing was conducted with detection of HPV E6 and E7 from mRNA HPV 16, 18, 31, 33, and 45 by real-time multiplex NASBA using the PreTect HPV-Proofer assay (NorChip AS, Klokkarstua, Norway) according to manufacturer's instructions. In order to avoid false negatives due to degradation of mRNA, primers and probes against human U1A mRNA are included in the PreTect HPV-Proofer kit as a performance and integrity control.

CINtec and p16/Ki-67 testing

The p16/Ki-67 dual staining was performed from the first container by Roche MTM Laboratories (Heidelberg, Germany) blinded to all study data. A second cytology slide was prepared from the residual PreservCyt material using a T2000 slide processor (Hologic). Immunostaining of anal cytology slides for p16/Ki-67 was performed using the CINtec PLUS Kit (Roche MTM Laboratories) according to the manufacturer's instructions. Slides that did not meet the squamous cellularity criteria as specified in the Revised Bethesda 2001 Cervical Cytology Classification system for reporting anal cytology were excluded from evaluation. A trained cytotechnologist reviewed all cases for the presence of cells staining positively with both markers. A case was considered positive if one or more squamous epithelial cell(s) stained both with a brown cytoplasmic stain (p16) and a red nuclear (Ki-67) irrespective of the interpretation of morphologic abnormalities. Slides without any double-stained cells were called negative for p16/Ki-67 dual-stain cytology.

Statistical analysis

Of the total of 363 men who were enrolled into the study, 339 (93.4%) had cytology results, 359 (98.9%) had valid Cobas results, 345 (95.0%) had valid PreTect proofer results, and 332 (91.5%) had valid p16/Ki-67 results. All assays were evaluated at the cutoffs specified by the manufacturers as outlined above. To evaluate clinical performance of the different assays, combined endpoints were used based on cytology and histology results to create four disease categories, analogous to what we previously used to improve misclassification of

cervical disease endpoints [16,17]: no dysplasia, including men with a nondysplastic biopsy or without a biopsy and with less than HSIL (<HSIL) cytology; anal intraepithelial neoplasia grade 1 (AIN1), including men with AIN1 histology and <HSIL cytology; AIN2, including men with AIN2 histology or with lower grade, normal, or no histology and with HSIL-AIN2 cytology; AIN3, including men with AIN3 histology or with lower grade, normal, or no histology and with HSIL-AIN3 cytology. Youden's index was calculated as sensitivity + specificity – 1. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value, with 95% confidence intervals and Youden's index, were calculated for two different endpoints, AIN2 and AIN3 (AIN2+) combined, and AIN3. Sensitivity and specificity with confidence intervals for all four biomarkers were plotted on a receiver operator characteristics (ROCs) curve graph. Statistical analyses were performed using Stata (StataCorp., College Station, Texas, USA) and summary ROC graphs were created using SigmaPlot (Systat Software San Jose, California USA).

Results

Anal cytology and histology, combined disease categories

The median age of men included in the study was 53 years, with an age range from 26 to 79 years. Most men were users of HAART (93%), with 89% having an HIV viral load less than 75 copies/ml and 97% a CD4 cell count higher than 200 cells/μl (82% higher than 350 cells/μl) at the time of enrollment. The majority of men (62%) reported that they were previously evaluated for anal cancer. Overall, 112 (30.8%) men had negative anal cytology results, 73 (20.1%) had ASC-US results, 27 (7.4%) had ASC-H results, and 60 (16.5%) had HSIL results. Anal biopsies were performed when lesions were observed during HRA; 70(19.3%) men did not have a biopsy in the study. Among men who received a biopsy, 85 (23.4%) had a normal histology or condyloma acuminata, 126 (34.7%) had AIN1, 55 (15.2%) AIN2, and 25 (6.9%) AIN3. Table 1 shows the cross-tabulation of cytology and histology results. We observed 28 patients with high-grade cytology results who had less than AIN2 (<AIN2) histology, including six men without a biopsy, two men without intraepithelial neoplasia in the biopsy, and 20 men with AIN1 in biopsy. To account for misclassification by anoscopy and biopsy placement, we used combined endpoints for the biomarker analyses resulting in four disease categories. One hundred and forty-eight (40.8%) men had no dysplasia (including men without a biopsy and <HSIL cytology), 106 (29.2%) had AIN1, 50 (13.8%) had AIN2 (including HSIL-AIN2 cytology), and 59 (16.3%) had AIN3 (including HSIL-AIN3 cytology).

Biomarker positivity in anal disease categories

Table 2 summarizes the positivity of carcinogenic HPV DNA testing, HPV16/18 DNA testing, HPV mRNA testing, and p16/Ki-67 testing, in the four anal disease categories. Two hundred and eighty-four of 359 (79.1%) men with HPV DNA test results were positive for carcinogenic HPV, including 59.9% without dysplasia, 84.5% with AIN1, and all (100%) with AIN2 or AIN3. One hundred and thirty-eight of 359 (38.4%) men were positive for either HPV16 or HPV18, constituting about half of the men positive for carcinogenic HPV DNA. There was a significant trend ($P < 0.001$) of increasing percentage of men testing positive for HPV16/18 with increasing severity of disease, from 13.4% of men without

dysplasia to 66.1% for men with AIN3. One hundred and seventy of 345 (49.3%) men were positive for HPVE6/E7 mRNA expression from HPV16, 18, 31, 33, and/or 45. There was a significant trend ($P < 0.001$) of increasing percentage of men testing positive for HPVE6/E7 mRNA for five carcinogenic types with increasing severity of disease, from 27.0% of men without dysplasia to 81% for men with AIN3. Two hundred and twenty of 332 (66.3%) men were positive for p16/Ki-67 double staining in anal cytology. There was a significant trend ($P < 0.001$) of increasing percentage of men testing positive for p16/Ki-67 double staining in anal cytology with increasing severity of disease, from 42.1% of men without dysplasia to 93.1% for men with AIN3.

Clinical performance characteristics

Table 3 summarizes clinical performance characteristics of the four markers at two cutoffs, AIN2 and greater and AIN3 restricted to 317 men who had results for all assays. For both endpoints, carcinogenic HPV testing was most sensitive (100% for AIN2+ and 100% for AIN3), followed by p16/Ki-67 (92.3% for AIN2+ and 93.0% for AIN3), HPVE6/E7 mRNA testing (79.8% for AIN2+ and 80.7% for AIN3), and HPV16/18 genotyping (62.5% for AIN2+ and 64.9% for AIN3). The specificity ranged from 22.7% for HR-HPV testing at the AIN3 cutoff to 74.2% for HPV16/18 genotyping at the AIN2+ cutoff. When ranking the tests by the maximal Youden's index, which gives equal weight to sensitivity and specificity, HPVE6/E7 mRNA testing ranked highest (Youden's index of 0.42 for AIN2+ and 0.36 for AIN3), followed by HPV16/18 genotyping (Youden's index of 0.37 for AIN2+ and 0.33 for AIN3), p16/Ki-67 staining (Youden's index of 0.36 for AIN2+ and 0.31 for AIN3), and HR-HPV testing (Youden's index of 0.28 for AIN2+ and 0.23 for AIN3). Figure 1 summarizes the sensitivity and specificity with confidence intervals for all four tests in the same graph for AIN2+ (Fig. 1a) and AIN3 (Fig. 1b). Positive predictive values for AIN2+ ranged from 40.3% for HR-HPV testing to 54.2% for HPV16/18 genotyping; for AIN3, positive predictive values ranged from 22.1% for HR-HPV testing to 30.8% for HPV16/18 genotyping (Table 3), reflecting the high disease prevalence in this population.

Combinations of biomarkers in disease categories

We evaluated the absolute risk of AIN2+ associated with combinations of mRNA testing, p16/Ki-67 testing, and HPV16/18 genotyping among 317 patients with results for all three assays (Table 4). The majority of men (96; 30.3%) were positive by all three tests, followed by men negative for all three tests (75; 23.7%). A large proportion of men were positive for p16/Ki-67, but negative for the other two tests (64; 20.2%). Forty-eight men (15.1%) were positive for mRNA and p16/Ki-67, but negative for HPV16/18 DNA. All other combinations were observed in 10 or fewer patients only. Participants who were positive for all three biomarkers had the highest risk of AIN2+ (61.5%), followed by men who were positive for mRNA and p16/Ki-67 (43.8%), but negative for HPV16/18 DNA. The risk in men who were positive for p16/Ki-67 only was 20.3%. The lowest risk was observed in men who were negative for all three tests (4.0%).

p16/Ki-67 testing at different cutoffs

Although the main analyses for p16/Ki-67 were conducted using a cutoff of one or more dual-stain positive cells that has been recommended in the evaluation of cervical cytology specimens, we also evaluated the performance of p16/Ki-67 at higher cutoffs (Table 5). Increasing the cutoff to two or more double-stained cells led to a minor decrease in sensitivity, but a substantial increase in specificity. A cutoff of five or more double-stained cells decreased the overall test positivity from 66 to 43%. Although the sensitivity for AIN2+ was significantly lower (77.6 vs. 92.3%) at this cutoff, the sensitivity for AIN3 remained unchanged (91.4 vs. 93.0%). The specificity increased significantly for both endpoints, resulting in the highest Youden's indices for all cutoffs (0.51 for AIN2+ and 0.58 for AIN3, respectively) and the highest Youden's index and PPV of all biomarkers analyzed.

Discussion

The risk of anal cancer is high among HIV-infected MSM and comparable to the risk of cervical cancer in the unscreened female population [18,19]. Infections with carcinogenic HPV genotypes have been identified as causal agents for most anal cancers. Anal intraepithelial neoplasia diagnoses, which are morphologically very similar to CIN, are common among HIV-infected MSM. In analogy to cervical cancer screening, identification and treatment of anal precancers may offer successful secondary prevention of anal cancers in high-risk populations [4]. Currently used screening options include HRA and anal cytology. Both require high standards of training, may have limited reproducibility, and may have limited sensitivity to detect anal precancers in widely implemented screening programs. Several biomarkers for HPV-related disease have been evaluated for cervical cancer screening, including markers of transforming HPV infections that can detect cervical precancers with good reproducibility, sensitivity, and specificity [10]. We sought to conduct phase 2 of a formal evaluation of biomarkers for use in anal cancer screening [20,21].

In a population of HIV-infected MSM, we evaluated the performance of several biomarkers for HPV-related carcinogenesis to detect anal precancers. The 363 men included in this study represent a typical clinic population of HIV-infected MSM who are on HAART, have a low viral load, and have a high CD4 cell count. The men were routinely followed for surveillance of AIDS-related diseases.

Overall, about 80% of men enrolled in the study were positive for carcinogenic HPV DNA and 30% had AIN2 or AIN3, figures very similar to what has been reported in a large meta-analysis of HPV infections and HPV-related disease in MSM, demonstrating the high disease burden in HIV-infected MSM [3].

For all four biomarkers analyzed, carcinogenic HPV DNA, HPV16/18 genotyping, HPVE6/E7 mRNA expression, and p16/Ki-67 staining, we observed an increase in positivity with increasing anal lesion grade. HPV16/18 genotyping had the lowest positivity in the population and increased from 13% in men without dysplasia to 66% in men with AIN3. HPVE6/E7 mRNA detection increased from 27% in men without AIN to 81% in men with AIN3 and p16/Ki-67 increased from 42% in men without AIN to 93% in men with AIN3. Carcinogenic HPV testing had the highest sensitivity and lowest specificity of all

biomarkers analyzed. The performance of type-restricted assays such as HPV16/18 genotyping and HPVE6/E7 mRNA expression strongly depends on the HPV genotype distribution in anal disease categories. About 66% of the AIN3 were related to HPV16/18 and about 80% were related to HPV16, 18, 31, 33, or 45. Consequently, p16/ Ki-67, which is not HPV genotype dependent, achieved higher sensitivity for AIN3 (93%), albeit with significantly lower specificity than HPV16/18 genotyping and HPVE6/E7 mRNA expression. When restricting men to those with infection with either HPV16, 18, 31, 33, or 45, HPVE6/E7 mRNA testing and p16/Ki-67 performed very similar (data not shown). When increasing the cutoff of p16/Ki-67 positivity to five or more double stain positive cells, the sensitivity for detecting AIN3 remained unchanged, whereas the specificity increased significantly, indicating that a higher threshold for p16/ Ki-67 cytology could lead to better risk stratification.

There are various applications for biomarkers in surveillance of HIV-infected MSM. Biomarkers could be used to identify men with a high risk of anal precancer to initiate further procedures such as HRA and biopsy. For these primary screening markers, a high sensitivity would be desirable; HPV DNA testing or p16/Ki-67 cytology may be suitable candidates. Especially p16/Ki-67 evaluated at a higher cutoff seems to offer a great tradeoff between sensitivity and specificity, with similar detection of AIN3, but significantly higher specificity compared with HR-HPV DNA testing. Performance of p16/Ki-67 at a higher cutoff needs to be evaluated in further studies. Another possible application for anal cancer screening biomarkers could be to identify lesions with highest risk of progressing to anal cancer to identify men that would most benefit from more aggressive diagnostic procedures and immediate treatment rather than expectant management. In analogy to CIN3, and based on the HPV genotype attribution in anal cancers, we assume that AIN3-related HPV16/18 has the highest risk of progressing to cancer. HPV16/18 genotyping or HPVE6/E7 mRNA expression for the five most carcinogenic types could be used as specific markers for lesions at highest risk of progression.

In general, our results demonstrate that biomarkers evaluated for cervical cancer screening can be implemented for surveillance of anal disease in HIV-infected MSM. Our study cannot address the question whether the risk of progression of AIN3 observed in this population is comparable to that of CIN3, thus warranting immediate treatment rather than expectant management. However, the positivity of the four assays was comparable to the corresponding cervical disease categories, suggesting that the functional disease steps up to precancer are very similar at the two sites [10].

The limitations of the study include the limited sensitivity of HRA to detect prevalent disease. We addressed this limitation by using composite endpoints of HRA and anal cytology, following a model previously established for cervical disease endpoints [16,17]. The specificity of all biomarkers increased substantially when using composite endpoints rather than histology endpoints alone, supporting the notion that there was disease misclassification based on HRA alone. Because cytology was used to help define the endpoint, we did not evaluate cytology as a screening test, as it would be invalid due to correlations between the test and endpoint.

The strengths of our study include a highly representative population of HIV-infected MSM, performing HRA on all study participants, a high biopsy rate, and an excellent liquid-based anal cytology that was used to improve the clinical endpoints. We performed rigorous sampling for biomarker assays [22] and evaluated the most promising biomarkers established for cervical cancer screening.

In summary, our study demonstrates that molecular features of anal disease categories are similar to those of corresponding cervical lesions. We compared four different cervical cancer biomarkers and showed that they may be useful for different purposes in anal cancer screening. Anal p16/Ki-67 cytology, especially when used at a cutoff higher than the recommended cutoff, may be useful as a primary screening test to decide who should be referred to HRA. Further studies evaluating biomarkers among HIV-infected MSM are warranted.

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References

1. Silverberg MJ, Lau B, Justice AC, Engels E, Gill MJ, Goedert JJ, et al. Risk of anal cancer in HIV-infected and HIV-uninfected individuals in North America. *Clin Infect Dis*. 2012; 54:1026–1034. [PubMed: 22291097]
2. Chaturvedi AK, Madeleine MM, Biggar RJ, Engels EA. Risk of human papillomavirus-associated cancers among persons with AIDS. *J Natl Cancer Inst*. 2009; 101:1120–1130. [PubMed: 19648510]
3. Machalek DA, Poynten M, Jin F, Fairley CK, Farnsworth A, Garland SM, et al. Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: a systematic review and meta-analysis. *Lancet Oncol*. 2012; 13:487–500. [PubMed: 22445259]
4. Wentzensen N. Screening for anal cancer: endpoints needed. *Lancet Oncol*. 2012; 13:438–440. [PubMed: 22445258]
5. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. *Lancet*. 2007; 370:890–907. [PubMed: 17826171]
6. Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE. Human papillomavirus testing in the prevention of cervical cancer. *J Natl Cancer Inst*. 2011; 103:368–383. [PubMed: 21282563]
7. Berry JM, Palefsky JM, Jay N, Cheng SC, Darragh TM, Chin-Hong PV. Performance characteristics of anal cytology and human papillomavirus testing in patients with high-resolution anoscopy-guided biopsy of high-grade anal intraepithelial neoplasia. *Dis Colon Rectum*. 2009; 52:239–247. [PubMed: 19279418]
8. Swedish KA, Lee EQ, Goldstone SE. The changing picture of high-grade anal intraepithelial neoplasia in men who have sex with men: the effects of 10 years of experience performing high-resolution anoscopy. *Dis Colon Rectum*. 2011; 54:1003–1007. [PubMed: 21730790]
9. Darragh TM, Winkler B. Anal cancer and cervical cancer screening: key differences. *Cancer Cytopathol*. 2011; 119:5–19. [PubMed: 21319310]
10. Sahasrabudde VV, Luhn P, Wentzensen N. Human papillomavirus and cervical cancer: biomarkers for improved prevention efforts. *Future Microbiol*. 2011; 6:1083–1098. [PubMed: 21958146]

11. Castle PE, Stoler MH, Wright TC Jr, Sharma A, Wright TL, Behrens CM. Performance of carcinogenic human papillomavirus (HPV) testing and HPV16 or HPV18 genotyping for cervical cancer screening of women aged 25 years and older: a subanalysis of the ATHENA study. *Lancet Oncol.* 2011; 12:880–890. [PubMed: 21865084]
12. De Vuyst H, Clifford GM, Nascimento MC, Madeleine MM, Franceschi S. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. *Int J Cancer.* 2009; 124:1626–1636. [PubMed: 19115209]
13. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, et al. The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA.* 2002; 287:2114–2119. [PubMed: 11966386]
14. Darragh TM, Tokugawa D, Castle PE, Follansbee S, Borgonovo S, Lamere BJ, et al. Interrater agreement of anal cytology. *Cancer Cytopathol.* 2012
15. Gage JC, Sadorra M, Lamere BJ, Kail R, Aldrich C, Kinney W, et al. Comparison of the cobas human papillomavirus (HPV) test with the hybrid capture 2 and linear array HPV DNA tests. *J Clin Microbiol.* 2012; 50:61–65. [PubMed: 22075592]
16. Wentzensen N, Schiffman M, Dunn ST, Zuna RE, Walker J, Allen RA, et al. Grading the severity of cervical neoplasia based on combined histopathology, cytopathology, and HPV genotype distribution among 1,700 women referred to colposcopy in Oklahoma. *Int J Cancer.* 2009; 124:964–969. [PubMed: 19030188]
17. Wentzensen N, Wilson LE, Wheeler CM, Carreon JD, Gravitt PE, Schiffman M, et al. Hierarchical clustering of human papilloma virus genotype patterns in the ASCUS-LSIL triage study. *Cancer Res.* 2010; 70:8578–8586. [PubMed: 20959485]
18. Palefsky J. Human papillomavirus-related disease in people with HIV. *Curr Opin HIV AIDS.* 2009; 4:52–56. [PubMed: 19339939]
19. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer.* 2010; 127:2893–2917. [PubMed: 21351269]
20. Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, et al. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst.* 2001; 93:1054–1061. [PubMed: 11459866]
21. Arbyn M, Ronco G, Cuzick J, Wentzensen N, Castle PE. How to evaluate emerging technologies in cervical cancer screening? *Int J Cancer.* 2009; 125:2489–2496. [PubMed: 19626591]
22. Gage JC, Ghosh A, Borgonovo S, Follansbee S, Wentzensen N, Gravitt PE, et al. A comparison of dacron versus Flocked nylon swabs for anal cytology specimen collection. *Acta Cytol.* 2011; 55:364–367. [PubMed: 21791907]

Fig. 1. Summary receiver operator characteristics plots of biomarker performance for detection of anal intraepithelial neoplasia 2+ (AIN2+) (a) and anal intraepithelial neoplasia 3 (AIN3) (b).

Table 1

Histology and cytology.

Cytology	Histology					
	No biopsy	Negative/condyloma acuminata	AIN1	AIN2	AIN3	Missing
Negative	35	40	27	7	2	1
ASC-US	9	21	32	6	5	0
ASC-H	6	5	6	9	1	0
LSIL	7	10	33	13	4	0
HSIL-AIN2	1	1	7	4	2	1
HSIL-AIN3	5	1	13	15	10	0
Missing	7	7	8	1	1	0
Total	70 (19.3%)	85 (23.4%)	126 (34.7%)	55 (15.2%)	25 (6.9%)	2 (0.6%)
						363

ACS-H, atypical squamous cells – cannot exclude high-grade lesion; ACS-US, atypical squamous cell of undetermined significance; AIN1, anal intraepithelial neoplasia grade 1; AIN2, anal intraepithelial neoplasia grade 2; AIN3, anal intraepithelial neoplasia grade 3; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

Table 2

Biomarker positivity in disease categories.

Test	No dysplasia	AIN1	AIN2	AIN3	Total	P trend
Cobas HR-HPV	88/147 (59.9%)	87/103 (84.5%)	50/50 (100%)	59/59 (100%)	284/359 (79.1%)	<0.001
Cobas HPV16/18	27/147 (13.4%)	41/103 (39.8%)	31/50 (62.0%)	39/59 (66.1%)	138/359 (38.4%)	<0.001
mRNA	38/141 (27.0%)	47/98 (48.0%)	38/48 (79.2%)	47/58 (81.0%)	170/345 (49.3%)	<0.001
p16/K1-67	56/133 (42.1%)	65/92 (70.7%)	45/49 (91.8%)	54/58 (93.1%)	220/332 (66.3%)	<0.001

Combined disease endpoints: no dysplasia, including men with a nondysplastic biopsy or without a biopsy and with <HSIL cytology; AIN1, including men with AIN1 histology and <HSIL cytology; AIN2, including men with AIN2 histology or with lower grade, normal, or no histology and with HSIL-AIN2; AIN3, including men with AIN3 histology or with lower grade, normal, or no histology and with HSIL-AIN3. P trend is based on the χ^2 -test. AIN1, anal intraepithelial neoplasia grade 1; AIN2, anal intraepithelial neoplasia grade 2; AIN3, anal intraepithelial neoplasia grade 3; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion.

Table 3

Clinical performance of biomarkers.

Test	Sensitivity	Specificity	PPV	NPV	Youden's index	Referral
Cobas HR-HPV						
AIN2+	100% (95.6–100)	27.7% (21.9–34.3)	40.3% (34.3–46.6)	100% (92.4–100)	0.277	81.39%
AIN3	100% (92.1–100)	22.7% (17.8–28.4)	22.1% (17.3–27.8)	100% (92.4–100)	0.227	
Cobas HPV16/18						
AIN2+	62.5% (52.4–71.6)	74.2% (67.7–79.8)	54.2% (44.9–63.2)	80.2% (73.8–85.4)	0.367	37.85%
AIN3	64.9% (51.1–76.8)	68.1% (62.0–73.6)	30.8% (22.9–40.1)	89.8% (84.5–93.5)	0.33	
mRNA						
AIN2+	79.8% (70.6–86.8)	62.4% (55.5–68.9)	50.9% (43.0–58.8)	86.4% (79.7–91.2)	0.422	51.42%
AIN3	80.7% (67.7–89.5)	55.0% (48.7–61.1)	28.2% (21.6–35.9)	92.9% (87.3–96.2)	0.357	
p16/Ki-67						
AIN2+	92.3% (85.0–96.4)	44.1% (37.4–51.1)	44.7% (37.9–51.6)	92.2% (84.7–96.3)	0.3643	67.82%
AIN3	93.0% (82.2–97.8)	37.7% (31.8–43.9)	24.7% (19.2–31.1)	96.1% (89.7–98.7)	0.307	

Combined disease endpoints: no dysplasia, including men with a nondysplastic biopsy or without a biopsy and with <HSIL cytology; AIN1, including men with AIN1 histology and <HSIL cytology; AIN2, including men with AIN2 histology or with lower grade, normal, or no histology and with HSIL-AIN2; AIN3, including men with AIN3 histology or with lower grade, normal, or no histology and with HSIL-AIN3. AIN1, anal intraepithelial neoplasia grade 1; AIN2, anal intraepithelial neoplasia grade 2; AIN3, anal intraepithelial neoplasia grade 3; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; NPV, negative predictive value; PPV, positive predictive value.

Risk of anal intraepithelial neoplasia grade 2 and anal intraepithelial neoplasia grade 3 according to combinations of mRNA, p16/Ki-67, and HPV16/18 DNA.

Table 4

Test combination	No dysplasia	AIN1	AIN2	AIN3	Total
mRNA ⁺ /p16/Ki-67 ⁺ /16/18 ⁺	12 (12.50%)	25 (26.04%)	25 (26.04%)	34 (35.42%)	96
mRNA ⁺ /p16/Ki-67 ⁺ /16/18 ⁻	16 (33.33%)	11 (22.92%)	11 (22.92%)	10 (20.83%)	48
mRNA ⁺ /p16/Ki-67 ⁻ /16/18 ⁺	3 (33.33%)	5 (55.56%)	0 (0%)	1 (11.11%)	9
mRNA ⁺ /p16/Ki-67 ⁻ /16/18 ⁻	5 (50.0%)	3 (30.0%)	1 (10.0%)	1 (10.0%)	10
mRNA ⁻ /p16/Ki-67 ⁺ /16/18 ⁺	2 (28.57%)	2 (28.57%)	2 (28.57%)	1 (14.29%)	7
mRNA ⁻ /p16/Ki-67 ⁺ /16/18 ⁻	26 (40.63%)	25 (39.06%)	5 (7.81%)	8 (12.50%)	64
mRNA ⁻ /p16/Ki-67 ⁻ /16/18 ⁺	2 (25.0%)	4 (50%)	1 (12.50%)	1 (12.50%)	8
mRNA ⁻ /p16/Ki-67 ⁻ /16/18 ⁻	61 (81.33%)	11 (14.67%)	2 (2.67%)	1 (1.33%)	75

Combined disease endpoints: no dysplasia, including men with a nondysplastic biopsy or without a biopsy and with <HSIL cytology; AIN1, including men with AIN1 histology and <HSIL cytology; AIN2, including men with AIN2 histology or with lower grade, normal, or no histology and with HSIL-AIN2; AIN3, including men with AIN3 histology or with lower grade, normal, or no histology and with HSIL-AIN3. AIN1, anal intraepithelial neoplasia grade 1; AIN2, anal intraepithelial neoplasia grade 2; AIN3, anal intraepithelial neoplasia grade 3; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion.

Table 5

Performance of p16/Ki-67 at different cutoffs.

Cutoff	Endpoint	Sensitivity	Specificity	PPV	NPV	Youden's index	Test positivity
p16/Ki-67, 2 cells	AIN2 +	88.8% (80.9–93.8)	55.4% (48.6–61.9)	48.7% (41.5–55.9)	91.2% (84.8–95.2)	0.44	58.9%
	AIN3	91.4% (80.3–96.8)	48.0% (41.9–54.1)	27.2% (21.2–34.1)	96.3% (91.2–98.6)	0.39	
p16/Ki-67, 5 cells	AIN2 +	77.6% (68.3–84.8)	73.2% (66.8–78.8)	58.0% (49.5–66.1)	87.2% (81.4–91.5)	0.51	43.2%
	AIN3	91.4% (80.3–96.8)	67.0% (61.1–72.5)	37.1% (29.3–45.6)	97.3% (93.6–99.0)	0.58	
p16/Ki-67, 50 cells	AIN2 +	47.7% (38.0–57.5)	90.6% (85.8–94.0)	70.8% (58.8–80.7)	78.4% (72.8–83.1)	0.38	21.8%
	AIN3	67.2% (53.5–78.6)	87.9% (83.3–91.4)	54.2% (42.1–65.8)	92.7% (88.6–95.4)	0.55	

AIN1, anal intraepithelial neoplasia grade 1; AIN2, anal intraepithelial neoplasia grade 2; AIN3, anal intraepithelial neoplasia grade 3; NPV, negative predictive value; PPV, positive predictive value.