Human Papillomavirus Genotypes in Anal High-Grade Squamous Intraepithelial Lesion (HSIL): Anal Intraepithelial Neoplasia Grades 2 (AIN2) and 3 (AIN3) Are Different

Jennifer M. Roberts[1], Isobel M. Poynten[2], Monica Molano[3,4], Dorothy A. Machalek[2,3,5], Richard J. Hillman[2,6], Patricia Guzman[1], Fengyi Jin[2], David J. Templeton[2,7,8], Christopher K. Fairley[9], Carmella Law[6], Suzanne M. Garland[3,4,10], Andrew E. Grulich[2], and Alyssa M. Cornall[3,4,10]

ABSTRACT

Background: Anal high-grade squamous intraepithelial lesion (HSIL) can be histomorphologically categorized into anal intraepithelial neoplasia (AIN) grade 2 (AIN2) and grade 3 (AIN3). Different risk factors for these two categories have been described. We investigated whether there were also differences in lesion-specific human papillomavirus (HPV) genotypes.

Methods: The Study of the Prevention of Anal Cancer (SPANC) recruited 617 gay and bisexual men (GBM); 36% of participants were HIV positive. At baseline, 196 men (31.8%) had histologic HSIL lesions. Tissue was available for genotyping in 171, with a total of 239 HSIL lesions (183 AIN3 and 56 AIN2). Using laser capture microdissection, each lesion revealed a maximum of one genotype.

Results: High-risk HPV (HR-HPV) genotypes were found in 220 (92.1%) HSIL lesions, with no significant difference between AIN3 (93.4%) and AIN2 (87.5%). AIN3 lesions had significantly more HPV16 (42.1%) than AIN2 lesions (12.5%; $P < 0.001$) and AIN2 lesions had significantly more non-16 HR-HPV types (75.0%) than AIN3 lesions (51.4%; $P = 0.002$). These associations were similar for HIV-negative men with HPV16 in 51.1% AIN3 and 18.2% AIN2 ($P = 0.001$) and non-16 HR-HPV in 40.0% AIN3 and 75.8% AIN2 ($P < 0.001$). For HIV-positive men, HPV16 remained more frequently detected in AIN3 (33.3% vs. 4.4% for AIN2; $P = 0.004$), but there was no difference between AIN3 and AIN2 for non-16 HR-HPV (62.4% vs. 73.9%; $P = 0.300$).

Conclusions: As HPV16 has the strongest link with anal cancer, the subcategorization of HSIL may enable stratification of lesions for anal cancer risk and guide anal HSIL management.

Impact: Stratification of anal cancer risk by histologic HSIL grade.

Introduction

Squamous cell carcinoma (SCC) of the anal canal has a strong causal relationship with chronic human papillomavirus (HPV) infection (1–5), particularly with HPV16 (6). HPV infection, specifically by one of the many "high-risk" or oncogenic mucosal subtypes (1, 2, 4) is a prerequisite for the cancer precursor lesion, high-grade squamous intraepithelial lesion (HSIL). In this regard, anal neoplasia has many biological correlates with cervical neoplasia, for which long-term successful screening programs exist in many countries (7, 8). These programs aim to detect and treat cervical HSIL to prevent its progression to SCC. Given the rising incidence of anal SCC, especially in several high-risk populations (9–11), similar anal screening programs have been proposed. Currently, there are several significant impediments to initiation of screening beyond research settings. One major problem is that a sensitive and specific screening test or test combination has not been identified (12). A second difficulty is a lack of evidence that treatment of anal HSIL prevents anal SCC (13). Furthermore, it is likely that not all HSIL lesions have equal malignant potential and it may be that treatment is indicated for only a small subset of HSIL lesions. Tools to stratify HSIL and subsequent management according to risk of malignant progression are needed.

Anogenital HSIL is defined by a set of histomorphologic features, most recently summarized by the Lower Anogenital Squamous Terminology (LAST) project (14). One of the expressed aims of the LAST project was to encourage use of diagnostic nomenclature, which reflects the current knowledge of HPV biology in the lower anogenital tract, namely that HPV produces two types of infection: productive infection causing a low-grade squamous intraepithelial lesion (LSIL) and transforming infection that results in HSIL. LAST allows for continued subcategorization of HSIL into anal intraepithelial neoplasia (AIN) grade 2 (AIN2) and grade 3 (AIN3), but does not mandate it. Use of this subdivision may enable investigation of whether such subclassification correlates with clinical outcomes, such as lesion resolution, persistence, and progression to cancer.

We have previously shown that HSIL-AIN3 and HSIL-AIN2 detected in gay and bisexual men (GBM) have different risk factors. In the Study of the Prevention of Anal Cancer (SPANC) at baseline, AIN3 was strongly associated with HIV positivity, more lifetime sexual

[1]Douglass Hanly Moir Pathology, Sydney, New South Wales, Australia. [2]The Kirby Institute, University of New South Wales, Kensington, Sydney, New South Wales, Australia. [3]Centre for Women's Infectious Diseases, The Royal Women's Hospital, Parkville, Melbourne, Victoria, Australia. [4]Molecular Microbiology, Murdoch Children's Research Institute, Parkville, Melbourne, Victoria, Australia. [5]School of Population and Global Health, University of Melbourne, Parkville, Melbourne, Victoria, Australia. [6]St Vincent's Hospital, Sydney, New South Wales, Australia. [7]Department of Sexual Health Medicine, Sydney Local Health District, Camperdown, Sydney, New South Wales, Australia. [8]Sydney Medical School, University of Sydney, Sydney, New South Wales, Australia. [9]Central Clinical School, Monash University, Melbourne, Victoria, Australia [10]Department of Obstetrics and Gynaecology, University of Melbourne, Parkville, Melbourne, Victoria, Australia.

Corresponding Author: Jennifer M. Roberts, Douglass Hanly Moir Pathology, 14 Giffnock Tce, Macquarie Park, Sydney, New South Wales 2113, Australia. Phone: 612-9855-5486; Fax: 612-9855-5177; E-mail: jroberts@dhm.com.au

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behaviors, and concurrent detection of anal canal HPV16. In contrast, AIN2 was associated with recent sexual exposures and detection of multiple anal canal high-risk HPV (HR-HPV) types (15). In separate longitudinal analyses, higher spontaneous clearance of AIN2 lesions compared with AIN3 lesions was observed (16). However, direct causality of specific HPV types with individual AIN2 and AIN3 lesions has not yet been shown. The ability to detect HPV types within individual histologic lesions using laser capture microdissection (LCM) enables detection of presumed causal HPV genotypes. Using LCM, we set out to further investigate the virologic differences between AIN2 and AIN3 lesions diagnosed at the baseline SPANC study visit (17, 18).

Materials and Methods

Participants

SPANC was a unique natural history study of anal HPV infection in GBM in Sydney, Australia. The study recruited GBM aged at least 35 years, both HIV positive and negative, predominantly from community settings in Sydney, Australia. The methodology has been previously described in detail (19). In brief, each participant had five clinic visits over a 3-year period. At each visit, men underwent a digital anorectal examination, anal swab sampling for cytology, and HPV testing and high-resolution anoscopy (HRA) with biopsy of lesions suspected of being HPV-associated.

Ethics approval for the SPANC study was granted by the St Vincent's Hospital (Sydney, Australia) Human Research Ethics Committee (File number 09/203). The study was conducted in accordance with the National Health and Medical Research Council National Statement of Ethical Conduct in Human Research 2007 and the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all participants prior to any study procedures being performed.

Histologic and virologic evaluation

This analysis is based solely on the virologic and histologic findings at baseline. Biopsies were processed in a standard manner and reported by one of three expert anogenital pathologists using LAST criteria and nomenclature, including the requirement for all AIN2 lesions to be $p16^{INK4a}$ (p16) positive. Each HSIL was classified as AIN2 or AIN3, based on whether the characteristic disordered proliferation of squamous cells involved more (AIN3) or less (AIN2) than two thirds of the epithelial thickness (14). For this study, all AIN3 lesions were also confirmed to be p16 positive. We used the LAST definition for p16 positivity and negativity (14). Strong and diffuse block staining for p16 is p16 positive. All other staining patterns are defined as p16 negative.

Tissue sections were fixed onto polyethylene naphthalate membrane slides for LCM. Individual cells from a well-characterized lesion were excised using the Veritas Microdissection instrument and DNA was extracted using the PicoPure DNA Extraction Kit (Life Technologies) into a final volume of 50 μL. Five microliters of this extracted DNA was tested for adequate quantity and quality using a quantitative PCR assay to detect a 110-bp fragment of the human beta-globin gene. A 10 μL aliquot of the original DNA extracted was used to detect the presence of HPV by using the RHA kit HPV SPF10-LiPA25, version 1 (Labo Bio-medical Products BV), which is able to identify HPV6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. Because of an inability to discriminate between HPV68 and 73 by the SPF10-LiPA25 assay (as their inner primer regions are identical), samples that were positive for HPV68/73 were tested on HPV68 and 73 type-specific qPCR assays targeting the E6 region as described previously (17). If a sample was HPV negative and beta-globin positive, it was tested for HPV by the DNA ELISA kit HPV SPF10, V.1. If the sample was ELISA positive, it was genotyped by using an RHA kit HPV SPF+ (Labo Bio-medical Products BV), which is able to identify HPV26, 30, 55, 61, 62, c64, 67, 69, 71, 71sub, 82, 83, 84, 85, 87, 89, 90, and 91. HPV controls provided by the manufacturer were included in each assay (17). The following HPV types were classified as "high risk": 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. HPV types 26, 34, 53, 67, 69, and 82 were classified as "possible high risk" (20, 21).

In general, only one biopsy was taken of a "lesion" at HRA, usually the clinically worst area. If there were 2 biopsies from the same octant and they had the same genotype, this was regarded as the same lesion for the purpose of our analysis. If two biopsies from the same octant had different genotypes, they were counted as two lesions.

Statistical analysis

We examined the prevalence of HPV (in the groups: HR-HPV; non-16 HR-HPV; HPV16; possible HR-HPV types) overall and by histology category. We also calculated HPV prevalence within each histology category, stratified by HIV status. We used the χ^2 test to examine differences between the groups, unless cell count was <5 in which case Fisher exact test was used. Statistical analyses were performed in Stata 15.1 (StataCorp).

Results

Baseline participant characteristics

Between 2010 and 2015, 617 participants were recruited, with a median age of 49 years (range 35–79 years). More than one third (36%) were HIV positive. At baseline, 196 (31.8%) had at least one histologic HSIL lesion, with 75 men having more than one HSIL lesion. For 171 (87.2%) of the 196 men, there was sufficient diagnostic tissue remaining for LCM and HPV genotyping after routine histologic reporting. These 171 men had 239 distinct HSIL (183 AIN3 and 56 AIN2) lesions analyzed.

HR-HPV genotyping results by HSIL grade and HIV status

Each HSIL was associated with a maximum of one HPV genotype. Overall, 20 different HPV genotypes were isolated from the HSIL lesions: 19 genotypes from AIN3 and 16 genotypes from AIN2. Fourteen of the 20 genotypes were HR-HPV and 6 genotypes were possible HR-HPV (**Fig. 1**). **Table 1** and **Figs. 2** and **3** present HPV types (HPV16, non-16 HR-HPV, and possible HR-HPV) detected in 239 individual HSIL by LCM, stratified by lesion grade and HIV status.

An HR-HPV genotype was found in 220 (92.1%) HSIL lesions, with no difference between AIN3 and AIN2 lesions (171, 93.4% and 49, 87.5%, respectively; $P = 0.150$). HPV16 was found in 77 (42.1%) of AIN3 and 7 (12.5%) of AIN2 lesions ($P < 0.001$). Conversely, non-16 HR-HPV types were found in 94 (51.4%) of AIN3 and 42 (75.0%) of AIN2 lesions ($P = 0.002$). Half of the 171 men (84, 49.1%) were HIV positive. HPV16 was found in 32 (27.6%) of all HSIL in HIV-positive men and 52 (42.3%) of all HSIL in HIV-negative men ($P = 0.017$). Non-16 HR-HPV types were found in 75 (64.7%) of all HSIL in HIV-positive men and 61 (49.6%) of all HSIL in HIV-negative men ($P = 0.019$).

In HIV-negative participants, HR-HPV genotypes were found in 82 (91.1%) AIN3 and in 31 (93.9%) AIN2 ($P = 1.00$). HPV16 was found in 46 (51.1%) AIN3 and 6 (18.2%) AIN2 ($P = 0.001$). Non-16 HR-HPV types were found in 36 (40.0%) AIN3 and 25 (75.8%) AIN2 ($P < 0.001$). In addition, HPV types classified as possible HR-HPV were detected in

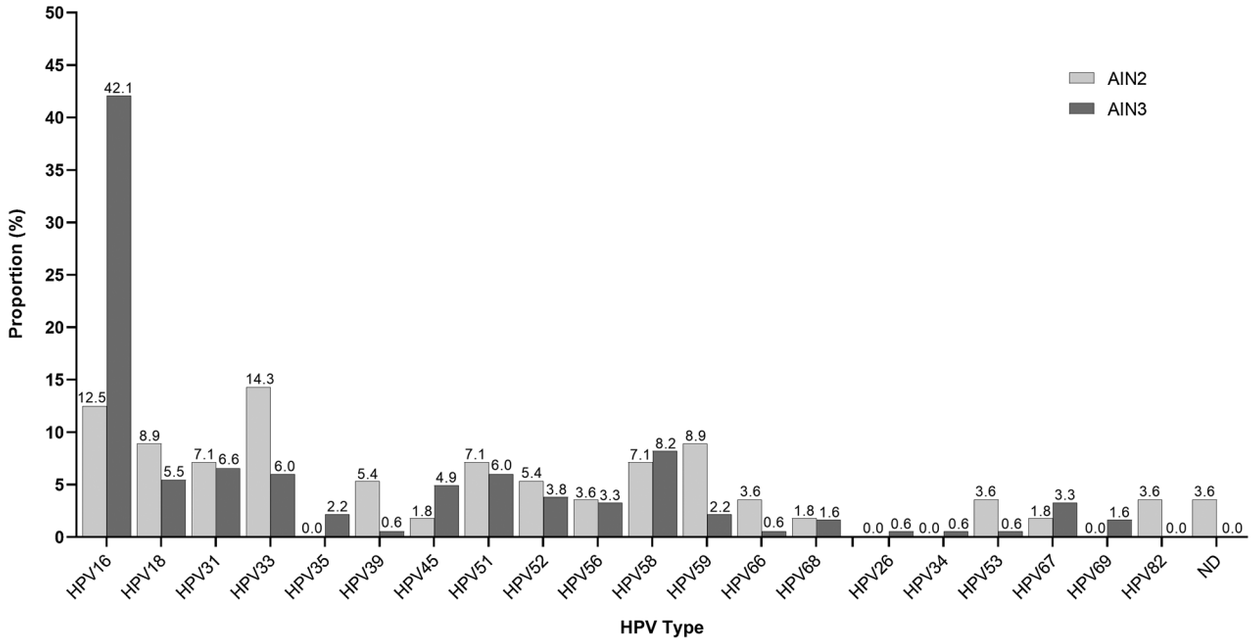


Figure 1.
Individual HPV types detected in 239 individual HSIL lesions, stratified by lesion grade.

8 (8.9%) AIN3 and 1 (3.0%) AIN2 ($P = 0.442$). Only one AIN2 lesion had no HPV detected (**Table 1**).

In HIV-positive participants, HR-HPV genotypes were found in 89 (95.7%) AIN3 and in 18 (78.3%) AIN2 ($P = 0.015$). HPV16 was found in 31 (33.3%) AIN3 and 1 (4.4%) AIN2 ($P = 0.004$). Non-16 HR-HPV types were found in 58 (62.4%) AIN3 and 17 (73.9%) AIN2 ($P = 0.300$). In addition, HPV types classified as possible HR-HPV were detected in 4 (4.3%) AIN3 and 4 (17.4%) AIN2 ($P = 0.048$; **Table 1**). Only one AIN2 lesion had no HPV detected.

Discussion

In this study of anal HPV infection and associated diseases in Australian GBM, HPV16 was the presumed causal HPV type in almost one in two HSIL-AIN3 lesions compared with one in eight AIN2 lesions. The HPV16 predominance in AIN3 was consistent for HSIL diagnosed in both HIV-negative (51.1% vs. 18.2%) and HIV-positive men (33.3% vs. 4.4%). Non-16 HR-HPV types were significantly more common in AIN2 lesions than in AIN3 lesions overall, and in lesions diagnosed in HIV-negative participants. Among HIV-positive participants, non-16 HR-HPV types were detected in similar proportions of AIN2 and AIN3 lesions.

The LAST Project guidelines (14) recognize that as an entity, AIN2 is likely to be a combination of high- and low-grade disease and classification is a subjective interpretation, prone to interobserver variability. In particular, at the "lower end" of AIN2, there is the likelihood of overlap with LSIL, which is characterized by disordered proliferation confined to the lower one third of the epithelial thickness.

Table 1. HPV types detected in 239 individual HSIL lesions, stratified by lesion grade and HIV status.

		N	Any HR-HPV *n* (%)	HPV16 *n* (%)	Non-16 HR-HPV *n* (%)	Possible HR-HPV types *n* (%)	HPV not detected *n* (%)
All	HSIL	239	220 (92.1)	84 (35.2)	136 (56.9)	17 (7.1)	2 (0.8)
	AIN2	56	49 (87.5)	7 (12.5)	42 (75.0)	5 (8.9)	2 (3.6)
	AIN3	183	171 (93.4)	77 (42.1)	94 (51.4)	12 (6.6)	0 (0.0)
	P[a]		*0.150*	*<0.001*	*0.002*	*0.546*	*0.054*
HIV negative	HSIL	123	113 (91.9)	52 (42.3)	61 (49.6)	9 (7.3)	1 (0.8)
	AIN2	33	31 (93.9)	6 (18.2)	25 (75.8)	1 (3.0)	1 (3.0)
	AIN3	90	82 (91.1)	46 (51.1)	36 (40.0)	8 (8.9)	0 (0.0)
	P[a]		*1.00*	*0.001*	*<0.001*	*0.442*	*0.268*
HIV positive	HSIL	116	107 (92.2)	32 (27.6)	75 (64.7)	8 (6.9)	1 (0.9)
	AIN2	23	18 (78.3)	1 (4.4)	17 (73.9)	4 (17.4)	1 (4.4)
	AIN3	93	89 (95.7)	31 (33.3)	58 (62.4)	4 (4.3)	0 (0.0)
	P[a]		*0.015*	*0.004*	*0.300*	*0.048*	*0.198*

Note: *P* values are italicized.
[a]*P* tests performed using χ^2 test unless cell count was <5 in which case Fisher exact test was used.

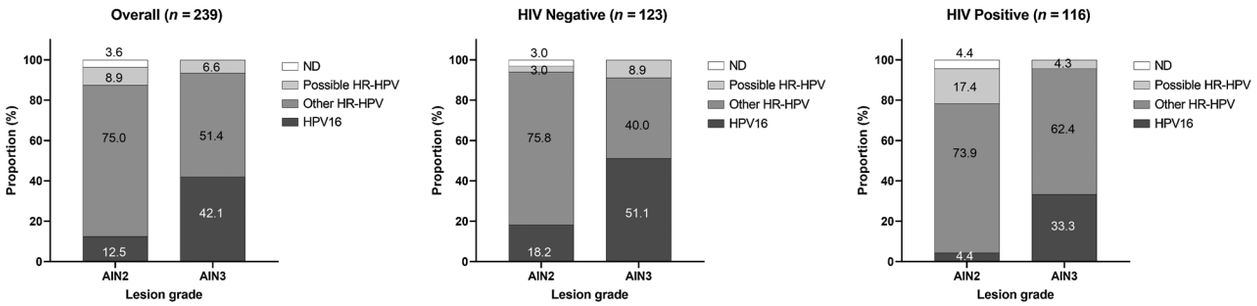
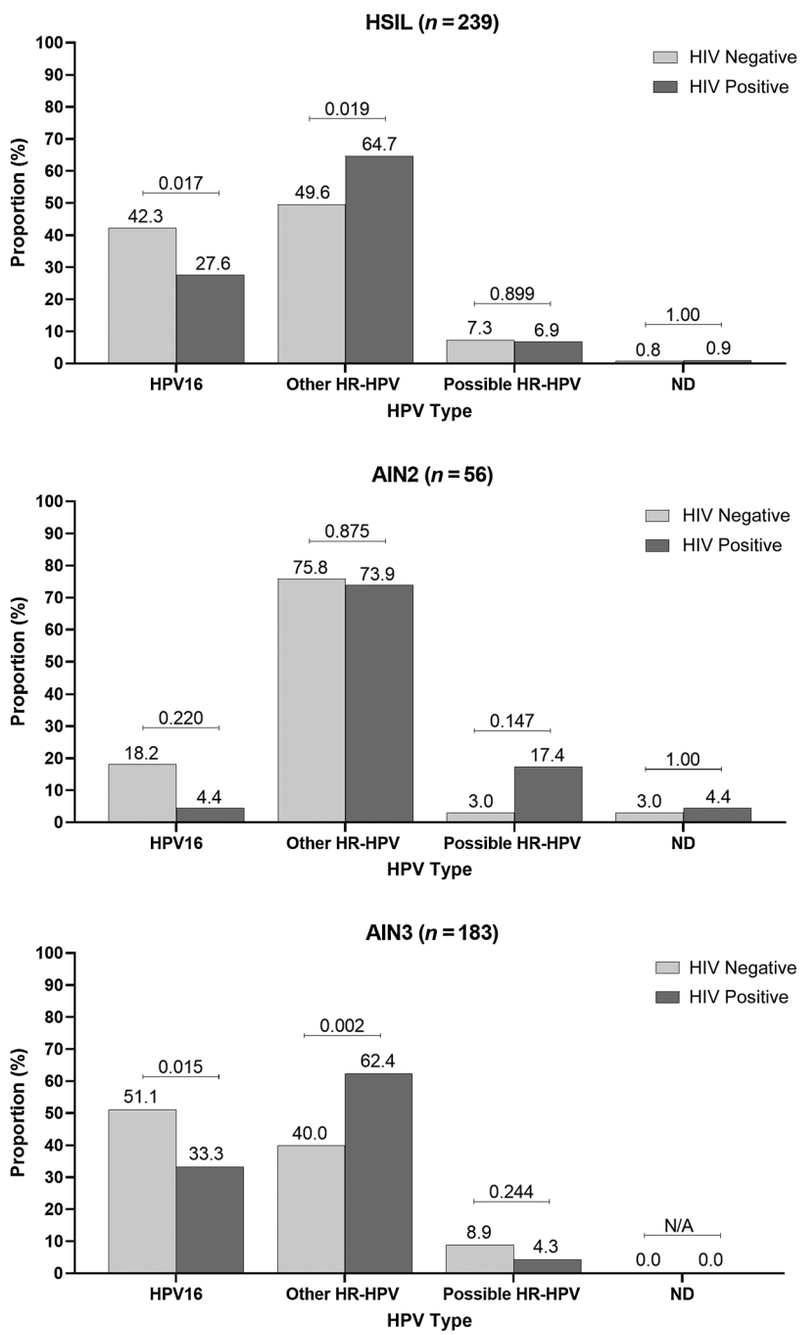


Figure 2.
HPV types detected in 239 individual HSIL lesions, stratified by lesion grade.

Figure 3.
HPV types detected in 239 individual HSIL lesions, stratified by HIV status.



This diagnostic boundary has been strengthened by LAST's evidence-based recommendation that any lesion considered on hematoxylin and eosin (H&E) stain to be AIN2, must also be p16 immunostain positive, to be confirmed as belonging in the HSIL category (14). Ultimately, the most important histologic differentiation is a diagnosis of HSIL-AIN3 or other, as HSIL-AIN3 is most like to be persistent and thus have higher cancer potential (16). Our results show a significant difference in presumed causal HPV types between AIN2 and AIN3 lesions. This indicates that even though there may be low grade disease within the HSIL-AIN2 category, the difference between the two subcategories is not solely morphologic.

We have previously shown in the SPANC study that lifetime sexual behaviors, positive HIV status, and HPV16 detection were strong predictors of a composite result (histologic ± cytologic) of AIN3, whereas composite AIN2 was associated with recent sexual behaviors and increasing types of anal canal HR-HPV (15). AIN2 and AIN3 were also found to differ in rates of spontaneous clearance, which further strengthens the evidence of a biological distinction between the lesion grades (16). Our current data build on this association, by confirming virologic differences between AIN2 and AIN3, most notably the strong association between HPV16, the most common cause of anal cancer, and AIN3 in both HIV-positive and HIV-negative men (6).

Earlier studies investigating HPV genotypes in anal HSIL have consistently shown that approximately 90% of lesions classified as HSIL are associated with HR-HPV types (1, 4, 22, 23). These studies used whole tissue genotyping, which would account for the detection of multiple HPV genotypes in biopsy tissue. Using LCM to exactly annotate the lesion allowed us to confidently detect a single genotype in each biopsy and this "one lesion one virus" concept has previously been reported by others (24). Two studies (4, 22) did subcategorize HSIL into AIN3 and AIN2 but as these studies predated LAST, their results are not directly comparable with ours. One study (22) reported that the proportion of biopsies containing HPV16 was similar in AIN2 and AIN3. The other study (4), similar to SPANC, reported that AIN3 was more likely to contain HPV16 than AIN2 but found no difference between immunocompetent and immunocompromised participants in the proportion of HSIL lesions associated with HPV16 (approximately 50%; ref. 4). In contrast, we found HPV16 to be more strongly associated with HSIL in the HIV negative than the HIV positive (42.3% vs. 27.6%). Consistent with our finding that HSIL lesions in HIV-positive men are more likely to be associated with non-16 HR-HPV than HPV16, a recently published meta-analysis showed that HIV-positive people have a higher proportion of HPV-associated anal and cervical cancers attributable to HR-HPV types other than HPV16 (6). This supports the notion that reduced immune function increases the likelihood that non-16 HR-HPV will cause HSIL lesions that would not occur in immune competent individuals.

A limitation of our study is that it is based on cross-sectional data collected at study baseline. As the study cohort underwent HRA± biopsy 5 times over a 3-year period, with careful documentation of lesion sites, we have the future potential to follow specific lesions over the course of the study. In further LCM genotyping studies, we will be able to report whether there are differences in HPV genotypes between transient (or regressing) lesions and persistent lesions, which may serve as a proxy for cancer risk.

Strengths of the study include use of the strict internationally accepted definition of HSIL, with subcategorization of AIN2 and AIN3, positive p16 staining in all lesions, and the ability to be certain of HPV genotype attribution by the use of LCM. Using LAST criteria, we have earlier shown good inter- and intraobserver reproducibility among the 3 reporting pathologists of SPANC (25).

In summary, within the category of HSIL, the histologic entity of AIN3 is more strongly associated with HPV16 than is AIN2. Coupled with the knowledge that HPV16 has the strongest association with persistence of anal HSIL (16) and with anal carcinoma (6), this heterogeneity within the HSIL category may enable stratification of anal cancer risk. Longitudinal studies are now needed to determine whether natural history of individual lesions correlates with lesion-specific HPV genotypes.

Disclosure of Potential Conflicts of Interest

J.M. Roberts reports other from Hologic Australia (ThinPrep consumables) during the conduct of the study and other from Roche Australia (donated antibodies for further research in this field) outside the submitted work. I.M. Poynten reports other from Seqirus (travel funding) outside the submitted work. M. Molano reports grants from Cancer Council Victoria (project: evaluating molecular biomarkers of anal cancer risk, application no. APP1130507) during the conduct of the study. D.A. Machalek reports nonfinancial support from MSD and Seqirus, grants from Seqirus, and other from Roche Diagnostics (manuscript license sponsorship) outside the submitted work. P. Guzman reports other from Hologic Australia (ThinPrep consumables) during the conduct of the study and other from Roche (donated antibodies for further research in this field) outside the submitted work. F. Jin reports grants from the National Health and Medical Research Council and Cancer Council New South Wales during the conduct of the study. C.K. Fairley owns shares in CSL Biotherapies. S.M. Garland reports grants (to institution for researcher-initiated grant for HPV young women's study), personal fees (lecture fees; work performed in personal time), and other from Merck (global advisory board member for HPV) outside the submitted work. A.E. Grulich reports grants from the National Health and Medical Research Council during the conduct of the study. A.M. Cornall reports grants from Cancer Council Victoria during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The views expressed in this publication do not necessarily represent the position of the Australian Government.

Authors' Contributions

J.M. Roberts: Conceptualization, resources, data curation, formal analysis, investigation, methodology, writing–original draft, writing–review and editing. **I.M. Poynten:** Formal analysis, supervision, validation, investigation, methodology, writing–original draft, writing–review and editing. **M. Molano:** Resources, data curation, formal analysis, validation, investigation, methodology, writing–review and editing. **D.A. Machalek:** Data curation, software, formal analysis, visualization, methodology, writing–review and editing. **R.J. Hillman:** Resources, supervision, project administration, writing–review and editing. **P. Guzman:** Data curation, writing–review and editing. **F. Jin:** Resources, data curation, software, writing–review and editing. **D.J. Templeton:** Resources, funding acquisition, writing–review and editing. **C.K. Fairley:** Writing–review and editing. **C. Law:** Resources, investigation, writing–review and editing. **S.M. Garland:** Resources, supervision, funding acquisition, investigation, project administration, writing–review and editing. **A.E. Grulich:** Conceptualization, resources, supervision, funding acquisition, investigation, project administration, writing–review and editing. **A.M. Cornall:** Conceptualization, resources, data curation, formal analysis, supervision, investigation, methodology, project administration, writing–review and editing.

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