

## Review

# Beyond p16 immunostaining: an overview of biomarkers in anal squamous intraepithelial lesions

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**Summary.** Histological grading of squamous intraepithelial lesions or intraepithelial neoplasia is fundamental for clinical management and for assessment of the risk of progression. Biomarkers are important for assisting correct grading of these lesions, reducing inter and intraobserver variability and most promising, for prognosis. Although p16 is the most studied biomarker in this setting, there are several other biomarkers that have been studied, reflecting also the need to find a better single or association option that can be more suitable, especially for classification purposes. A PubMed and Embase search was conducted from their inception until April 2018, aiming to identify biomarkers evaluated in histological samples of anal squamous intraepithelial lesions, other than p16. Information on “Ki-67”, “ProEx™ C”, “p53”, “human papillomavirus L1 capsid protein”, “stathmin-1”, “minichromosome maintenance protein”, “p21”, “proliferating cell nuclear antigen”, “histones”, “human papillomavirus E4”, “chromosomal abnormalities” and “methylation” was collected and reviewed. From these, the most studied biomarker was by far Ki-67. In many cases there were few studies performed for each biomarker, with no clear standardized interpretation of the immunostaining. An increased positive rate with more severe grades of lesions was shown in many cases. Prognostic data are limited and need to be further validated.

**Key words:** Anal squamous intraepithelial lesions, Anal intraepithelial neoplasia, Biomarkers, Immunohistochemistry, Methylation

## Background

A unified histological classification currently exists for all anogenital sites regarding squamous intraepithelial lesions or intraepithelial neoplasia (-IN), including low-grade (low-grade squamous intraepithelial lesions LSIL) and high-grade lesions (high-grade squamous intraepithelial lesions HSIL) (Darragh et al., 2012). This classification is clinically relevant, high-grade lesions have a higher squamous cell carcinoma (SCC) progression rate. For these two groups of lesions different therapeutic and follow-up strategies are advocated.

There is important interobserver and intraobserver variability in squamous intraepithelial lesions grading. This agreement has been shown to be better for the diagnosis of carcinomas and normal tissue, than for other grades (Carter et al., 1994). Biomarkers have an important role in reducing this variability (Walts et al., 2008; Bean et al., 2009) and can be very helpful in the histological classification (Darragh et al., 2012). Some studies, especially in cervical neoplasia, have shown that biomarkers can have a possible prognostic value (Ozaki et al., 2001; Negri et al., 2004; Yoshida et al., 2008; Del Pino et al., 2009; Hoshikawa et al., 2010; Cortecchia et al., 2013; Liao et al., 2014), although more data is needed. In some cases, these data can also give insight to possible physiopathological alterations implicated in the

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carcinogenesis.

In 2012, the Lower Anogenital Squamous Terminology (LAST) (Darragh et al., 2012) recommended p16 immunostaining to be used in specific situations of squamous intraepithelial lesions grading: 1) distinguishing precancer (-IN2 and -IN3) and a mimic of precancer; 2) for grading clarification (precancer vs. LSIL) in H&E morphologic interpretation of -IN2; 3) disagreement in precancerous lesions interpretation and the differential diagnosis includes a precancerous lesion; and 4)  $\leq$ IN 1 lesion and a high-risk patient for a missed HSIL. Although there are several advantages of p16 use in this setting, the possibility of having false positive results and subsequent overtreatment or false negative results and undertreatment exists, minimised by specific guidelines for using it (Darragh et al., 2012). A systematic review and meta-analysis (Albuquerque et al., 2018) on p16 positivity in anal squamous intraepithelial lesions (ASIL)/anal intraepithelial neoplasia (AIN) showed that 7% of all anal LSIL (including AIN1/LSIL/condyloma), 12% of LSIL/AIN1 (excluding condylomas), 76% of AIN2 and 90% of AIN3 were positive. More data on p16 immunostaining as a prognosis marker is needed and there are no current recommendations for using it as a prognostic biomarker for anogenital cancer progression (Darragh et al., 2012).

There are several other biomarkers that have been studied, reflecting also the need to find a better single or an association option that can be more suitable, especially for a classification purposes. The most recent review on this topic (Pirog, 2015) is almost exclusively focused on genital lesions, and in immunohistochemistry and *in situ* hybridization techniques. Anal SCC incidence is increasing (Smittenaar et al., 2016) thereby creating the need for more updated information on anal premalignant lesions.

This overview will summarize the published information on these biomarkers in ASIL or AIN, other than p16.

## Methods

A PubMed and Embase search was conducted from their inception until April 2018, including the terms “anal carcinoma in situ”, “anus neoplasm”, “anal intraepithelial neoplasia”, “anal squamous intraepithelial lesions”, “anal dysplasia”, “anal precursors lesions” and “biomarkers”. The same terms were also used and “biomarkers” was replaced by adding specific terms like “Ki-67”, “ProEx™ C”, “p53”, “human papillomavirus L1 capsid protein”, “stathmin-1”, “minichromosome maintenance protein”, “p21”, “proliferating cell nuclear antigen”, “histones”, “human papillomavirus E4”, “chromosomal abnormalities” and “methylation”. Reference lists of retrieved articles were also reviewed to identify other relevant studies. Only studies that assessed biomarkers in histological samples were considered. All included articles were written in English. Studies that were published only in an abstract form or

case reports were also not considered. Studies involving biomarkers in anal SCC without concomitant ASIL or that only evaluated p16 staining were excluded. Anal squamous intraepithelial lesions and AIN, LSIL and AIN1, HSIL and AIN2/3 were used synonymously; the classification used in each study was normally adopted and described accordingly.

A table including all the studies that were published involving these biomarkers is presented (Table 1). The description includes the first author, year of publication, number and histological classification of samples and the most important results.

## Results and discussion

### Ki-67

Ki-67 is a proliferation marker not directly related to human papillomavirus (HPV) infection that is present in all phases of the cell cycle including G1, S, G2, and mitosis, but not in G0 or the quiescent phase (Bean et al., 2007). After p16, this is the most studied marker in ASIL (Calore et al., 2001a,b; Mullerat et al., 2003; Manzione et al., 2003; Walts et al., 2006; Bean et al., 2007; Kreuter et al., 2007; Scarpini et al., 2008; Walts et al., 2008; Bean et al., 2009; Walker et al., 2009; Pirog et al., 2010; Kreuter et al., 2010; Bala et al., 2013; Cotter et al., 2014; Kreuter et al., 2016; Larson et al., 2016; Hissong et al., 2018; Leeman et al., 2018).

Studies are very heterogeneous in the definition of Ki-67 positivity, making comparisons and conclusions difficult to establish. This is particularly true for the cut-off percentage used to determine a relevant result of Ki-67 positive nuclei, which is highly variable among different studies. Positive Ki-67 staining is exclusively nuclear and there is a reported increased expression with the increasing severity of anal dysplastic lesions (Manzione et al., 2003; Mullerat et al., 2003; Walts et al., 2006; Bean et al., 2007; Kreuter et al., 2010). Some studies have also shown that the distribution is different according to the grading, LSIL is usually confined to the lower one-third of the epithelium, whereas HSIL are distributed in the middle and/or upper one-third (Calore et al., 2001b; Mullerat et al., 2003; Bean et al., 2007). Regarding prognosis evaluation in ASIL, there was one study that positively correlated the percentage of Ki-67 positive cells in LSIL (in anal condyloma) from HIV-positive patients with the recurrence of these lesions before one year of follow-up, following condyloma excision and cauterization (Calore et al., 2001a).

This immunostaining also seems to reduce intraobserver and interobserver variability (Walts et al., 2008) in ASIL interpretation, although there are some conflicting results (Bean et al., 2009). Ki-67 staining combined with p16 was not recommended by LAST (Darragh et al., 2012) due to the minimal improvement in sensitivity and specificity. It should be considered as a single biomarker when p16 is inconclusive or technically inadequate (Darragh et al., 2012). Its major limitation is

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that false positive results can be seen in cases with increased inflammation, reactive changes and with tangential sectioning (resulting in the appearance of positive nuclei) (Bean et al., 2007; Pirog et al., 2010; Bala et al., 2013). This is important when applying Ki-67 in the differential diagnosis between squamous intraepithelial lesions from reactive lesions. A negative staining may be useful for excluding dysplastic lesions, but positive results in isolation should be interpreted

with caution (Bean et al., 2007).

### ProEx™ C

ProEx™ C is an immunohistochemical marker that contains monoclonal antibodies against minichromosome maintenance protein 2 (MCM2) and DNA topoisomerase II $\alpha$  (TOP2A). These are cell-cycle-related proteins implicated in early S phase. Its up-regulation

**Table 1.** biomarkers studied in anal squamous intraepithelial lesions or anal intraepithelial lesions.

Biomarker	Reference*	Description of the anal/perianal samples	Aims/outcome	Most important results regarding this biomarker(s)
Ki-67	Calore et al., 2001a	Total samples 38, total patients 38. All condyloma with LSIL.	Expression of Ki-67 in LSIL in HIV-positive patients to predict recurrence of these lesions.	Patients with LSIL recurrence had a higher percentage of Ki-67 positive cells.
	Calore et al., 2001b	Total samples 34, total patients 34. Condyloma with low-grade (n=25) and condylomas with high-grade dysplasia (n=9).	Expression of Ki-67 in anal condyloma from HIV-patients and association with AIN grade.	Condylomas with low-grade dysplasia had a lower percentage of Ki-67 positive cells compared with condylomas with high-grade dysplasia.
	Manziona et al., 2003	Total samples 97, total patients 97 (only 43 with Ki-67 staining). Condyloma with low-grade (n=79) and condyloma with high-grade AIN (n=18). For Ki-67 staining condyloma with low-grade (n=35) and condyloma with high-grade AIN (n=8).	Evaluation of the post-operative follow-up of HIV-patients with anal condyloma, relating recurrence to their HIV status. Ki-67 staining was done in the anal samples.	Ki-67 expression was higher in high-grade than low-grade AIN, but there was no association with HIV status.
	Walts et al., 2006	Total samples 104, total patients 74. Normal (n=37), condyloma and AIN 1 (n=26), AIN2 (n=25), AIN3 (n=16).	Expression of p16 and Ki-67 in AIN diagnosis and grading.	Ki-67 positivity expression in >50% of the squamous cell nuclei increased with the severity of AIN.
	Bean et al., 2007	Total samples 75, total patients 55. Normal (n=35), condyloma (n=12), AIN 1 (n=14), AIN 2 (n=25), AIN3 (n=16).	Expression of p16 and Ki-67 in AIN diagnosis and grading.	The expression of Ki-67 was positively correlated with the degree of anal dysplasia. The sensitivity and specificity of Ki-67 for high-grade AIN was 71% and 84%, respectively.
	Walts et al., 2008	Total samples 60, total patients 52. Normal (n=25), condyloma and AIN 1 (n=12), AIN 2 (n=9), AIN3 (n=14).	Evaluation of p16 and Ki-67 in reducing inter and intraobserver variability in the diagnosis and grading of AIN.	p16 and Ki-67 decreased intra- and interobserver variability in the diagnosis and grading of AIN.
	Bean et al., 2009	Total samples 77, total patients 57. Normal (n=25), AIN1 (n=28), AIN2 (n=3), AIN3 (n=13). No consensus classification in 8 samples.	Evaluation of p16 and Ki-67 in improving interobserver agreement in diagnosing AIN.	p16 alone improved interobserver agreement and was better than H&E, Ki-67, and H&E/p16/Ki-67 combined.
	Walker et al., 2009	Total samples 226, total patients 118. Condyloma (n=31), AIN1 (n=47), AIN2 (n=36), AIN3 (n=64) and SCC (n=48).	Evaluation of growth factor receptors, p16 and Ki-67 expression and distribution in AIN and SCC in HIV-positive and HIV-negative patients.	HIV-positive patients had a higher Ki-67 expression in all grades (except AIN1), in comparison with HIV-negative patients. This was statistically significant for AIN2 and AIN3.
	Pirog et al., 2010	Total samples 75, total patients 75. Normal (n=17), fibroepithelial polyps (n=14), condyloma (n=6), AIN1 (n=11), AIN2 (n=16), AIN3 (n=11).	Expression of p16 and Ki-67 in improving diagnostic accuracy in AIN.	Ki-67 detected (all) anal HPV-related changes with a high degree of sensitivity (100%) and specificity (100%). p16 staining was strongly associated with high-grade AIN.
	Cotter et al., 2014	Total samples 60, total patients 60. Condyloma (n=18), AIN1 (n=7), AIN2 (n=5), AIN3 (n=6), SCC (n=24). Staining was performed in 18 cases with dysplasia AIN1-AIN3.	Evaluation of the classification system for diagnosing and grading AIN. Role of p16 and Ki-67 for a better grading system.	Ki-67 staining did not appear to add discriminating value for AIN grading. All AIN grades showed some Ki-67 positivity.
Kreuter et al., 2016	Total samples 60 (staining only performed in 59, three samples with staining were penile intraepithelial neoplasia, anal samples with staining 56). Non-dysplastic condyloma (n=22), AIN1 (n=5), AIN2 (n=12, staining in 11), AIN3 (n=17), SCC (n=1).	Evaluation of anal condylomas with different grades of dysplasia. p16 and Ki-67 staining were performed.	HPV-induced anogenital lesions of high-risk patients (e.g., HIV-positive MSM) should be evaluated histopathologically, including p16 and/or Ki67 immunohistochemical analysis.	

has been evaluated in several cervical studies, mainly involving cervical precancerous lesions (Shi et al., 2007; Badr et al., 2008; Pinto et al., 2008; Conesa-Zamora et al., 2009). An association between high-risk HPV (Badr et al., 2008; Conesa-Zamora et al., 2009) and more severe grades of cervical dysplasia (Badr et al., 2008; Conesa-Zamora et al., 2009) was described.

There are two studies that evaluated this marker in ASIL/AIN. In both cases comparisons with p16 and Ki-67 were made. In one study (Bala et al., 2013), ProEx™ C was more sensitive than p16 in detecting anal lesions that harboured high-risk HPV DNA (94% vs. 83%, respectively), but p16 was more specific (90% vs. 59%, respectively) with fewer false-positive results in anal LSIL with low-risk HPV (false-positive cases in p16 were 5% and 75% in ProEx™ C). A positive result was considered when a strong nuclear staining extending into the upper one third of the epithelium and involving at least one half of the lesion was present. A negative result

was defined as a strong nuclear staining confined to the lower one third of the epithelium or a weak nuclear staining. In another study (Larson et al., 2016), ProEx™ C staining was tested alone and in combination with p16 and Ki-67 in the diagnosis and grading of AIN. An absence of staining or staining limited to the basal epithelial layer was recorded as negative, other nuclear staining's regarded as positive. ProEx™ C full-thickness alone had higher sensitivity (80%) for differentiated AIN 2/3 from AIN 1, but with the lowest specificity (74%). p16 staining alone for AIN2/3 diagnosis comparing with AIN1 diagnosis, had a lower sensitivity (40%), but higher specificity (98%). Although combining ProEx™ C with Ki-67 or p16 increased its specificity (83% and 100%, respectively), it lowered the sensitivity (68% and 28%, respectively) for distinguishing AIN2/3 from AIN1, especially when associated with p16.

In these anal studies, it has been suggested that there is a higher sensitivity and lower specificity of this

**Table 1.** (Continued).

ProEx™ C and Ki-67	Bala et al., 2013	Total of samples 75, total of patients 65. Non-dysplastic lesions (n=17), 23 LSIL/condyloma (n=23), 20 HSIL/AIN2-3/CIS (n=20), 15 invasive SCC (n=15).	Performance of p16, ProEx™ C and Ki-67 in AIN and anal SCC, and correlation with HPV genotypes.	ProEx™ C was more sensitive than p16 in detecting anal lesions that had high-risk HPV DNA (94% vs. 83%, respectively). p16 was more specific (90% vs. 59%, respectively) with fewer false-positive results, especially in anal LSIL with low-risk HPV.
	Larson et al., 2016	Total of samples 67, total of patients 44. Non-dysplastic (n=20), AIN 1 (n=22), AIN 2/3 (n=25).	Performance of ProEx™ C alone and combination with p16 and Ki-67 in the diagnosis and grading of AIN.	ProEx™ C full-thickness alone had higher sensitivity (80%) and lower specificity (74%) for differentiated AIN 2/3 vs. AIN 1. Combined with Ki-67 or p16 increased the specificity (83% and 100%, respectively), but lower sensitivity (68% and 28%, respectively) for differentiated AIN2/3 from AIN1.
MCM, p21, PCNA and Ki-67	Kreuter et al., 2007	Total samples 21, total patients 21. AIN1 (n=6), AIN2 (n=5), AIN3 (n=10).	Expression of p16, Ki-67, MCM, PCNA, p21 before and after 16weeks of imiquimod treatment. Correlation with high-risk HPV DNA load.	Ki-67, MCM and PCNA expression declined significantly after therapy (but not p21). There was not a significant correlation with high-risk HPV DNA load.
	Scarpini et al., 2008	Total samples 54, total patients 54. Normal (n=3), AIN1 (n=20), AIN2/3 (n=22), SCC (n=9)	Expression of MCM 2, MCM5, Ki-67 in anal samples and comparison with anal cytology.	Increased expression of MCM 2 and 5 was associated with the increasing severity of AIN. Sensitivity for AIN2/3 was 84%, for AIN1/viral changes was 76%, with an overall specificity (for any lesion) of 77%.
	Kreuter et al., 2010	Total samples 49, total patients 49. Normal mucosa (n=17), AIN1 (n=8), AIN2 (n=10), AIN3 (n=14).	Expression of MCM3, MCM4, MCM6, MCM7, p21, Ki-67, p16, PCNA in diagnosing high-grade anal lesions.	Sensitivity and specificity of PCNA and p21 for high-grade diagnosis were lower than for the other biomarkers (100%). For a cut-off of 50% positive cells, p21 sensitivity was 42% and specificity was 44%. For a cut-off of 25% positive cells, PCNA sensitivity was 92% and specificity was 29%.
HPV L1-capsid protein	Hernandez et al., 2011	Total samples 68, total patients 36. Normal mucosa (n=11), HSIL (n=26), SCC (n=31).	Expression of HPV L1-capsid protein in the progression of anal high-grade lesions to anal SCC.	HPV L1-capsid protein expression was only identified in HSIL. The expression was lower in HSIL associated with a concomitant invasive SCC vs. isolated HSIL.
	Patil and Yang, 2015	Total samples 145, total patients 100. Condylomas (n=34), LSIL (n=64), HSIL (n=47).	Combined utility of HPV L1-capsid protein and p16 in assessing anal squamous intraepithelial lesions.	HPV L1 nuclear expression was higher in low-grade than high-grade lesions. Simultaneous HPV L1 and diffuse p16 staining were frequently mutually exclusive.
Stathmin-1 and Ki-67	Hissong et al., 2018	Total samples 40, total patients 40. Normal mucosa (n=10), AIN1 (n=10), AIN2 (n=10), AIN3 (n=10).	Expression of stathmin-1 in AIN and comparison with Ki-67 and p16 staining.	A staining extending above the lower one-third of the epithelial thickness favoured high-grade AIN. Specificity 100% and sensitivity 80% for high-grade AIN (p16 sensitivity was 100%).

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biomarker when compared to p16. Nonetheless, in studies involving cervical precancerous lesions, different conclusions were obtained, namely the higher sensitivity of p16 (Shi et al., 2007). Similar to Ki-67, a major limitation of ProEx™ C is related to the possibility of false-positive results in cases with increased chronic inflammation (Bala et al., 2013), immature squamous metaplasia and regenerating epithelium (Pirog, 2015). The LAST consensus (Darragh et al., 2012) did not find sufficient evidence to recommend its use, except in cases where p16 was inconclusive or technically inadequate. In this setting, this biomarker and/or Ki-67 could be considered.

### *Minichromosome maintenance protein, p21, proliferating cell nuclear antigen*

Minichromosome maintenance proteins (MCM) are identified during proliferative phases of the cell cycle and are essential for DNA replication. The MCM protein family consists of 6 major isoforms (MCM 2-7) (Kreuter et al., 2010). They are markers of proliferation, only present in the cell cycle and lost from the cell during quiescence and differentiation (Freeman et al., 1999). There are several studies exploring MCM in cervical intraepithelial neoplasia (CIN) and cervical SCC (Williams et al., 1998; Mukherjee et al., 2007). In

**Table 1.** (Continued).

	Ogunbiyi et al., 1993	Total samples 126, total patients ND. Normal mucosa (n=39), AIN 1 (n=14), AIN 2 (n=7), AIN 3 (n=20), AIN 3 specimens adjacent to invasive disease (n=12), SCC (n=34).	Expression of p53 in anal squamous intraepithelial lesions and association with HPV16 DNA.	Nuclear p53 positivity was higher in high-grade AIN/cancer.
	Walts et al., 1993	Only 4 anal samples, LSIL (n=3), SCC (n=1)	Localization of p53 and HPV in precancerous and invasive lesions anogenital squamous lesions.	A higher immunoreactivity for p53 was not seen for HSIL vs benign lesions/LSIL. In HSIL, p53 was expressed in individual nuclei at various levels of the abnormal epithelium and in the basal layer of the adjacent epithelium. In LSIL it was limited to the basal layer of the epithelium.
p53 (one study also Ki-67)	Mullerat et al., 2003	Total samples 78, total patients 78. Normal mucosa (n=8), anal warts (n=20), LSIL (n=12), HSIL (n=27), SCC (n=11).	Expression of p53 and Ki-67 in precancerous and invasive anal lesions.	Expression of both biomarkers increased as the lesions became more dysplastic and invasive.
	Roma et al., 2008	Total 117 samples, total patients 51. Normal mucosa (n=34), squamous hyperplasia (n=5), AIN (n=64) and SCC (n=14).	Expression of 14-3-3σ, p16 and p53 in AIN and anal SCC.	Expression of p53 and 14-3-3 σ were not present in the normal mucosa, only in AIN and anal SCC. p16 and p53 were mutually exclusive. Expression of 14-3-3σ was independent of immunoreactivity status for p16 and p53.
	Hui et al., 2017	Total samples 55, total patients 55. AIN2 (n=15), AIN3 (n=33), SCC (n=7).	Identification of HSIL and anal SCC incidentally discovered on haemorrhoidectomy specimens. High-risk-HPV genotyping, p53 and p16 expression in these lesions.	Frequent overlap between p53 staining and HPV positivity, particularly when HPV 31 was identified.
Histones	Hu et al., 2016	Total samples: 41, total patients 41. AIN 1 (n=4), AIN 2 (n=7), AIN 3 (n=16) and anal SCC (n=14)	Expression of macroH2A2 in AIN and anal SCC.	Loss of macroH2A2 expression was associated with the progression of anal neoplasm (AIN3 and anal cancer). Patients with AIN with macroH2A2-negative lesions showed earlier recurrence than those with macroH2A2-positive lesions.
HPV E4 and Ki-67	Leeman et al., 2018	Total samples: 67, total patients 54. Normal (n=17), AIN1 (n=15), AIN2 (n=20) and AIN3 (n=15) samples.	Expression of HPV E4, p16 and Ki-67 in AIN.	Ki-67 was helpful in discriminating AIN samples vs. normal (sensitivity 92%, specificity 100%). E4+/p16+ staining showed that most AIN2 are different from transformed AIN3. 70% of AIN2 are E4+ vs. 0% of AIN3 (sensitivity 70%, specificity 100%)
Chromosomal changes	Haga et al., 2001	Total samples 30, total patients 30. AIN1 (n=4), AIN2 (n=17), AIN3 (n=9).	Genetic changes in AIN samples from HIV-positive and HIV-negative patients.	There were more genetic changes in higher degrees of AIN. The most common was a gain in chromosome arm 3q.
	Gagne et al., 2005	Total samples 46, total patients 44. Normal (n=5), condyloma (n=2), AIN1 (n=7), AIN2 (n=10), AIN3 (n=17), unknown grade (n=3), colonic tissue (n=2).	Evaluation of AIN samples to determine genomic alterations and correlation with HPV DNA.	Higher grades of AIN showed more genomic alterations than lower grades.
	Ricciardi et al., 2014	Total samples 52, total patients 52. Normal mucosa (n=16), LSIL (n=12), HSIL (n=15), SCC (n=9).	Evaluation of chromosome 3q26 gain in anal squamous intraepithelial lesions and anal SCC.	Chromosome 3q26 gain was only present in SCC and HSIL. It was associated with recurrent dysplasia in patients with HSIL.

normal cervical epithelium, MCM were only present in the basal proliferative compartment (Williams et al., 1998; Mukherjee et al., 2007) and in CIN they were present in the epithelial layers. MCM2 is part of ProEx™ C, previously described.

Proliferating cell nuclear antigen (PCNA) is involved in DNA repair, DNA methylation, cell cycle regulation and chromatin remodelling and is a marker of cell proliferation. Its overexpression has been shown in dysplastic cervical epithelium, with higher expression in higher grades of CIN and in cervical SCC (Heatley, 1998; Maeda et al., 2001; Astudillo et al., 2003; Wang et al., 2004; Wang et al., 2006).

p21 is a cyclin-dependent kinase inhibitor, member of the Cip/Kip family, involved in DNA damage repair and apoptosis. p21 has been described as having an “antagonistic duality” because it often inhibits apoptosis, but also has antiproliferative effects (Gartel and Tyner, 2002).

The expression of these markers has been evaluated during 16 weeks of imiquimod treatment of 21 AIN and perianal intraepithelial neoplasia lesions in HIV-positive MSM and related to the course of high-risk HPV DNA load during therapy (Kreuter et al., 2007). Only distinct nuclear staining was considered positive. Eighteen (86%) patients had a complete histological clearance of AIN after imiquimod. Ki-67, MCM and PCNA expression declined significantly after therapy (but not p21 expression), although it did not significantly correlate with high-risk HPV DNA load. This study had a very small sample size and treatment of patients with AIN 1 (n=6) was also done. In another study (with a larger sample, n=49) (Kreuter et al., 2010), MCMs (3, 4, 6 and 7), Ki-67 and PCNA (but not p21) expression did correlate with cumulative lesion high-grade HPV-DNA

load. Sensitivity and specificity of PCNA and p21 for HSIL diagnosis were lower than the rest of the biomarkers. The expression of MCM (MCM 2 and 5) was also shown to increase in the progression from normal anal epithelium through AIN to SCC (Scarpini et al., 2008).

There is no current formal recommendation for using these biomarkers in a first line approach to squamous intraepithelial lesions' grading.

#### HPV L1 capsid protein

HPV L1 capsid protein is important to promote viral entry capacity and was recognized as a therapeutic target. Currently available HPV vaccines target this protein. It represents 90% of the total protein in the surface of the virus (Rauber et al., 2008). There are several studies that have evaluated the expression of this protein in cervical carcinogenesis showing that this could be a marker of low-grade disease and low-risk HPV infection (Yoshida et al., 2008; Lee et al., 2008), as well as a prognostic marker. Low-grade CIN lesions that are HPV L1 capsid antigen negative seem to progress more commonly to high-grade (Griesser et al., 2004), while HPV L1 capsid positive regressed more (Rauber et al., 2008). This may be due to the loss of HPV L1 capsid occurring after viral integration (Lee et al., 2008; Hernandez et al., 2011) leading to ineffective stimulation of immune responses favouring viral survival (Yoshida et al., 2008). Higher levels of HPV L1 capsid appear in the productive/replicative phase of the HPV life cycle, and correlate with the development of proliferative lesions, like LSIL (Patil and Yang, 2015).

There are two studies evaluating HPV L1 capsid protein in ASIL. In a study by Hernandez et al. (2011),

**Table 1.** (Continued).

	Zhang et al., 2005	Total samples 184, total patients 76. Normal mucosa (n=57), LSIL (n=74), HSIL (n= 41), SCC (n=12)	DNA methylation abnormalities in anal squamous intraepithelial lesions and anal SCC.	DNA methylation was more common in anal SCC and HSIL. Methylation of IGSF4 and DAPK1 was only present in anal SCC and HSIL.
	Wiley et al., 2005	Total samples 16, total patients 16 (with HPV 16 infection). LSIL (n=5), HSIL (n=11)	Methylation of CpG in in anal squamous intraepithelial lesions from HIV-positive men.	Methylation of CpG site at nucleotides 31, 37, 43, 52, or 58 was associated with an increased risk of HSIL.
DNA methylation	Hernandez et al., 2012	Total samples 29, total patients 24. Normal mucosa (n=3), HSIL (n=11), anal SCC (n=15).	Methylation profiles in anal squamous intraepithelial lesions and anal SCC.	In the progression from normal anal mucosa and HSIL to invasive SCC, 22 CpG loci representing 20 genes showed significant differential methylation.
	Molano et al., 2016	Total samples 52, total patients 26. 26 paired WTS and LCM samples, including LSIL (n=3) and HSIL (n=23).	Methylation CpG of HPV genome of in anal squamous intraepithelial lesions.	In HSIL a higher percentage of methylation at CpG site 37,52 and 58 was shown.
	Lorincz et al., 2017	Total samples 148, total patients 148. Normal (n=30), LSIL (n=43), HSIL (n=59 anal +11 perianal), SCC (n=5).	Evaluation of DNA methylation patterns of HPV and EPB41L3.	Methylation of HPV16 and EPB41L3 were associated with increasing severity of in anal squamous intraepithelial lesions and with anal cancer.

AIN: anal intraepithelial lesions, CIS: carcinoma in situ; HPV: human papillomavirus; HSIL: high-grade squamous intraepithelial lesions; LCM: laser capture microdissection; LSIL: low-grade squamous intraepithelial lesions; MCM: minichromosome maintenance proteins; MSM: men who have sex with men; ND: non-described; PCNA: proliferating cell nuclear antigen; PIM: papillary immature metaplasia; SCC: squamous cell carcinoma; WTS: whole tissue sections.

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HPV L1 capsid nuclear staining, located in the superficial squamous epithelium, was identified in 38% (10/26) of HSIL, but not in normal anal mucosa or SCC. In HSIL associated with a concomitant invasive SCC, only 15% (2/13) demonstrated nuclear L1 expression as compared to 62% (8/13) of isolated HSIL ( $p=0.002$ ). Authors hypothesized that nuclear expression of L1 may be lost in the progression of anal HSIL to SCC and may serve as a possible marker of worse prognosis. There were some major limitations of this study, related to the lack of a LSIL comparison group and the relatively small sample size ( $n=68$ ). High-grade squamous intraepithelial lesions HPV L1 capsid staining was higher than that described in the cervix (Yoshida et al., 2008) and other anal studies (Patil and Yang, 2015).

More recently, Patil and Yang (2015) studied the expression of the L1 capsid protein and p16, alone or in association, and evaluated their significance as biomarkers for ASIL. HPV L1 nuclear staining (restricted to the superficial half of the squamous epithelium) was present in 68% (23/34) of condylomas, 52% (32/62) of LSILs and 9% (4/46) of HSILs ( $p<0.0001$ ). Simultaneous HPV L1 and diffuse p16 staining patterns were mutually exclusive in all cases except four (12%) presenting focal HSIL in a background of extensive LSIL.

In cervical carcinogenesis studies (Yoshida et al., 2008) it seems that L1(-)/p16(+) cases have the potential for progression, whereas L1(+)/p16(-) and L1(-)/p16(-) cases may be non-progressive lesions or potentially in remission (Hoshikawa, 2008). The last situation may be due to the fact that the loss of L1 capsid protein expression may be related to integration of viral DNA into the host genome, but also to a latent infection with low or no synthesis of HPV oncoprotein and no HPV production. A pathway with increasing severity of cervical lesions for L1(-)/p16(-), L1(+)/p16(-), L1(+)/p16(+), and L1(-)/p16(+) was proposed (Hoshikawa et al., 2008; Yoshida et al., 2008). More studies are needed regarding the combination of these two markers for the assessment of squamous intraepithelial lesions progression/regression potential.

### *Stathmin-1*

Stathmin-1 or oncoprotein 18 is a microtubule-destabilizing phosphoprotein (Belmont and Mitchinson, 1996) that has an important role in the control of mitosis. It has been shown to be overexpressed in multiple types of cancers, especially in advanced, invasive and metastatic cancer (Belletti and Baldassarre 2011). There are some data on this biomarker in CIN (Howit et al., 2013) showing an increased expression with more advanced histological grades and a higher specificity for CIN3 than p16 immunostaining.

Regarding ASIL, there is only one study (Hissong et al., 2018) comparing immunohistochemistry for stathmin-1, p16, and Ki-67. High-grade and low-grade lesions/normal mucosa had different distribution of

staining. A staining extending above the lower one-third of the epithelial thickness favoured high-grade AIN. There were no cases of extension above this limit in normal or low-grade lesions and 60% (6/10) of AIN2 and 80% (8/10) of AIN3 had this pattern. The sensitivity for the diagnosis of high-grade AIN was 70%, specificity was 100%, positive predictive value of 100%, and negative predictive value of 77%. Despite the high specificity, the sensitivity was lower than p16 for high-grade lesions (100%).

More studies, with larger sample sizes exploring combinations with other markers are needed, not only for ASIL, but also for squamous intraepithelial lesions in other sites.

### *p53*

p53 is a tumor suppressor protein that stops cell cycle during G1 phase in response to DNA replicative errors (Pirog, 2015). Mutations have been described in several types of cancers (Nigro et al., 1989; Hollstein et al., 1991). The accumulation of unregulated and mutated p53 can be detected by immunohistochemistry (Pirog, 2015).

There are several studies investigating the immunohistochemical expression of p53 in AIN. In two studies (Ogunbiyi et al., 1993; Mullerat et al., 2003), an increased p53 expression in more severe grades of AIN were shown. This increased expression was not obtained in all studies involving anogenital samples (Walts et al., 1993), although the staining distribution was different according to the grade. For HSIL, it was expressed in individual nuclei at various levels of the abnormal epithelium and in the basal layer of the adjacent epithelium. For LSIL it was limited to the basal layer of the epithelium.

14-3-3 $\sigma$  is a p53-regulated G2/M inhibitor, previous research implicated this marker in vulvar carcinogenesis (Gasco et al., 2002; Wang et al., 2008) and there is also a study in anal carcinogenesis (Roma et al., 2008). Positive staining was defined as >10% of cells labelled with antibodies against p53 or 14-3-3 $\sigma$ ; in p53 only nuclear staining was considered. Samples from normal anal mucosa, AIN and anal SCC were used, but no grading of AIN was provided. Expression of p53 and 14-3-3 $\sigma$  was not present in the normal mucosa, just in AIN and anal SCC.

Studying p53 was especially relevant for a better understating of the pathophysiology of several precancerous lesions and cancer. Besides p16 and Ki-67, this is one of the most frequently described immunohistochemical biomarkers in ASIL, although most of the studies were done several years ago (Ogunbiyi et al., 1993; Walts et al., 1993; Mullerat et al., 2003; Roma et al., 2008). There are some recent studies that still consider it useful for evaluating these neoplastic lesions. In a recent study by Hui et al. (2017), p53 (and p16) staining in high-grade lesions and SCC incidentally discovered on haemorrhoidectomy specimens was used.

### Histones

MacroH2A is a variant of a core histone that has been suggested to have tumour suppressor function (Rodríguez-Paredes and Esteller 2011; Cantariño et al., 2013). The H2A family includes mH2A1 (splice variants mH2A1.1 and 1.2) and mH2A2.

The histone  $\gamma$ -H2AX has been studied in cervical and vulvar precancerous lesions (Leventakos et al. 2017; Brustmann et al. 2011), but this was not the case for histone variant macroH2A2 expression that, to our knowledge, was only studied in AIN (Hu et al., 2016). The reduction of macroH2A2 is more common than macroH2A1 in human tumour samples (Hu et al., 2016). A loss of histone variant macroH2A2 expression was associated with progression of AIN, by immunohistochemistry analysis (nuclear staining), in one study (Hu et al., 2016). All cases of AIN 1 and AIN 2 had macroH2A2 expression. Expression was lost in 38% (6/16) of AIN3 and 71% (10/14) of anal SCC ( $p=0.002$ ). In a 5-year follow-up of AIN, macroH2A2-negative lesions ( $n=6$ ) showed earlier recurrence ( $p=0.017$ ) when compared with AIN macroH2A2-positive lesions ( $n=21$ ), after treatment (excision and ablation). In this study, AIN2 sample results were similar to low-grade lesions and not to high-grade lesions. This was a small study and there are no other studies that have replicated this possible tumour suppressive function of macroH2A2 in anal neoplasia. For prognosis evaluation, the sample numbers in both groups were quite small. There is no current evidence favouring any role in grading, but macroH2A, as a possible prognostic marker (of early recurrence, like in the study described), should be further studied and validated.

### HPV E4

HPV E4 is implicated in HPV genome amplification and virus synthesis. It can be detected in biopsies by immunostaining (Doorbar, 2013) and was described as a possible marker for initiation of the productive phase of the HPV life cycle (Leeman et al., 2018). It has been evaluated in CIN (Griffin et al., 2015; van Baars et al., 2015), alone and in combination with other biomarkers, like MCM and, especially p16 (Griffin et al., 2015; van Baars et al., 2015)

There was a study evaluating HPV E4, (and also Ki-67 and p16) in ASIL, including 17 normal, 15 AIN1, 20 AIN2 and 15 AIN3 samples (Leeman et al., 2018). None of the normal biopsies were E4+, 53% of the AIN1 were E4+, 70% of the AIN2 were E4+ and none of the AIN3 were positive. Focal (upper quarter of the epithelium) and extensive (upper half of the epithelium or more) E4 expression were both considered positive results. Ki-67 was helpful discriminating between AIN samples from normal samples (positivity above the lower one third of the epithelium) and p16 for AIN2/3 vs. AIN1/normal samples. E4 was useful in discriminating AIN2 from

AIN3, although this did not seem to be a biomarker for the conventional histological grading purposes. AIN2 seemed to be a heterogeneous group of lesions, different from transformed AIN3 (E4-/p16+), showing both entry into productive HPV infection and transforming activity (in most cases E4+/p16+). Similar findings were described in the cervix (Griffin et al., 2015; van Baars et al., 2015). These data also questioned the simplicity of a 2-tiered classification system (Darragh et al., 2012).

### Chromosomal abnormalities

Chromosomal changes have been more extensively studied in cervical and in vulvar carcinogenesis. The most common one is DNA copy number gain in the long arm of chromosome 3 (Heselmeyer et al., 1996; Matthews et al., 2000; Jee et al., 2001; Allen et al., 2002; Bryndorf et al., 2004; Huang et al., 2005) and evidence favours more alterations in cancers and high-grade lesions (Heselmeyer-Haddad et al., 2003, 2005; Caraway et al., 2008; Seppo et al., 2009). Previous studies in the cervix also evaluated the role of 3q gain in predicting the progression from low to high-grade disease (Heselmeyer-Haddad 2005). One of the most studied abnormalities is the chromosome band 3q26 and the two most common implicated genes in this chromosome are human telomerase RNA gene (TERC) and PIK3CA. TERC is associated with enhancing telomerase activity that regulates telomere length (Heselmeyer-Haddad et al., 2003, 2005). PIK3CA is implicated in the PI 3-kinase/AKT signalling pathway, which is involved in regulating cell growth and apoptosis. In cervical cancer it has been shown that the increase of PIK3CA is associated with high kinase activity, leading to proliferation and decreased apoptosis (Ma et al., 2000).

Studies that evaluated genetic alterations in ASIL (three studies) showed similar findings to cervical disease and suggested a possible similar mechanism. In a study using comparative genomic hybridization (CGH) (Haga et al., 2001), more severe grades of AIN were associated with more genetic changes. The most common regional DNA copy number change was gain in chromosome arm 3q, although other genetic changes were present in AIN, mainly in chromosomes 6q, 20q and Xq. The gain of chromosome arm 3q in HSIL was also shown in another study (Gagne et al., 2005) using microarray-based CGH. Higher grades of AIN showed more genomic aberrations than lower grades. There were other common regions of gain on chromosome arms 1p, 1q, 8p, and 20q; the most common regions of loss were on chromosome arms 2q, 7q, 11p, 11q, and 15q.

In a more recent retrospective study (Ricciardi et al., 2014), using fluorescence in situ hybridization (FISH) technique, chromosome 3q26 gain was present in 78% of SCC (7/9), 53% (8/15) of HSIL and in none of LSIL (0/12) and non-dysplastic (0/16) cases. A positive result was considered if there was a copy number of 3q >4 in at least 2 nuclei. The sensitivity for HSIL or cancer was 58%, with



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a specificity of 100%, positive predictive value was 100% and negative predictive value was 62%. Moreover, 3q26 gain was associated with more recurrent dysplasia (mean follow-up was 3 years). Due to the higher positive predictive value, a positive result may help confirm HSIL, although a negative result cannot exclude it.

### DNA methylation

DNA methylation is defined by the addition of a methyl group to cytosine nucleotides. Abnormal promoter DNA methylation leading to reduced gene expression and involving tumor suppressor genes has been implicated in several tumours (Baylin et al., 1998; Jones and Laird 1999; Esteller et al., 2001; Herman and Baylin 2003). Promoter-hypermethylation events have been considered to be some of the most promising cancer markers (Herman and Baylin 2003).

In anal precancerous and invasive lesions, results have been similar to those obtained in cervical lesions (Yang et al., 2004; Li et al., 2005). The first studies involving anal samples were conducted over ten years ago (Wiley et al., 2005; Zhang et al., 2005). In a study by Zhang et al. (2005) real-time methylation-specific PCR analysis of 11 genes DAPK1, IGSF4, MLH1, HIC1, RARB, p14, TP73, MGMT, RASSF1, APC, and CDKN2A was done (these genes were already previously evaluated in cervical cancer). DNA methylation was more common in anal SCC and HSIL than in LSIL and normal mucosa. Methylation of IGSF4 and DAPK1 was only present in anal SCC and HSIL. The methylation profile of anal biopsies was similar to anal cytology, which is important when considering a screening program. In another study (Hernandez et al., 2012), similar results were obtained for CpG loci with DAPK1, but not for IGSF4. In the progression from normal anal mucosa and HSIL to invasive SCC, 22 CpG loci representing 20 genes showed significant differential methylation ( $p=0.01$ ).

Studies on DNA methylation of HPV genome in anal lesions are scarce (Wiley et al., 2005; Molano et al., 2016). Methylation of CpG site at nucleotides 31, 37, 43, 52, or 58 was associated with an increased risk of HSIL (Wiley et al., 2005; Molano et al., 2016). Recently, Lorincz et al. (2017) found an association between methylation of HPV16 and EPB41L3 with increasing severity of AIN and anal cancer. EPB41L3 is a tumour suppressor gene that inhibits cell proliferation and promotes apoptosis.

These are considered promising biomarkers also for squamous intraepithelial lesions, but more evidence and studies that directly compare methylation with other biomarkers (like p16) are needed.

### Conclusions

Cervical carcinogenesis has been more widely studied, including in the role of biomarkers in diagnosis, grading and prognosis of precancerous lesions. Studies

including anal precancerous lesions and biomarkers are scarce when compared to cervical, and largely try to mimic these cervical studies. Natural history or prognostic data are available in many studies of cervical HPV-related disease, but such follow-up is rarely available in the anus/perianus, leading to a common extrapolation of the findings in the cervix to other anogenital sites.

Currently only p16 immunostaining is recommended, in specific situations, for anogenital squamous intraepithelial lesions histological grading, although validation as a prognostic biomarker is lacking. Considering other biomarkers, Ki-67 is the most studied, but it is not currently recommended alone or in combination with p16. It can be an option when p16 is inconclusive or technically inadequate (as for ProEx™ C). DNA methylation is considered promising for anogenital squamous intraepithelial lesions, but more evidence and comparison with other biomarkers are needed. Stathmin-1 and HPV E4 are the newest biomarkers that have been evaluated in ASIL/AIN.

Several biomarkers have been evaluated in ASIL, but unfortunately, in most cases there were few studies performed for each with no standardized interpretation of the immunostaining. An increased positive rate with more severe grades of lesions was shown in many cases; prognostic data are limited and need to be further validated. Prognostic biomarkers are one of the most important and necessary fields of research, especially for patient's progression risk stratification from LSIL to HSIL and HSIL to cancer.

The fact that there were multiple markers studied or currently under study, reflects the importance and need for this information as a tool for clinical evaluation, but also that we are probably still far from finding an ideal biomarker.

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