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Immunohistochemistry in the Diagnosis of Squamous Intraepithelial Lesions of the Uterine Cervix

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1. Introduction

During the last few decades accumulated epidemiological, clinical, and experimental evidence has revealed the important role of human papillomavirus (HPV) in the development of cervical carcinomas, an association almost unique in cancer epidemiology. Several important questions have been answered and a large number of scientific studies have paved the way for the introduction of new and effective vaccines, which will hopefully diminish the incidence of HPV-related carcinomas and precursor lesions in forthcoming years (Crum et al., 2003; zur Hausen, 1977, 2008). However, the exact recognition and proper treatment of clinically important lesions often poses problems both to pathologists and gynecologists.

Morphology remains the gold standard for lesion diagnosis, despite the fact that it can be hampered by inter- and intra-observer variability. Additionally, the contribution of morphology in the field of human papillomavirus research cannot be overemphasized, since cytologic and/or histologic examination allow the recognition of viral cytopathic effects, and, with the aid of immunohistochemical and other *in situ* techniques, may reveal the exact cells, in which some main interactions take place. Thus, the correlation of cellular alterations with new sensitive methods of detection either for human papillomavirus nucleic acids or for HPV-related intracellular interactions might lead both to the identification of different groups of lesions according to their clinical significance, as well as to the correct application of current morphological criteria.

The following chapter will focus on those immunohistochemical methods that can facilitate or confirm the detection of intraepithelial lesions of cervical squamous epithelium (SILs) in biopsy specimens, additionally presenting in brief some data concerning the mechanisms by which these specific cellular targets are related to important actions of HPV oncoproteins. Finally, a short comment concerning the application in diagnosis of methods other than immunohistochemistry has been added.

1.1 HPV in carcinogenesis

In the last three decades a large number of scientific studies have focused on the subject of cervical carcinogenesis. These resulted in the accumulation of data linking several types of

human papillomavirus to the development of cervical cancer (Bosch et al., 2002; Bosch et al., 2006; Crum et al., 1984; zur Hausen, 1977, 2009). The revealed strong association led to the suggestion that HPV is not only the main cause of cervical cancer, but also a necessary cause (Walboomers et al., 1999). Human papillomavirus is associated with more than 99% of all cervical cancer cases. In addition, a significant percentage of vulvar, vaginal, penile, anal and perianal carcinomas are HPV-positive (Fuste et al., 2010; Gross & Pfister, 2004; Insinga et al., 2008; Munoz et al., 2006), often containing HPV 16 DNA (zur Hausen, 2009), while a fraction of carcinomas in other sites of the human body has also been linked to high-risk (HR) HPV infections. Percentages of HPV positivity observed in carcinomas of the anogenital region are presented in Table 1.

Human papillomavirus is estimated to comprise a causal agent in 5% of human cancers and is associated with more human cancers than any other virus (Bergonzini *et al., 2010)*. Among them, cervical cancer represents a well-studied prototype of a human tumor related to a viral infection.

Vaginal carcinomas	60-91%
Vulvar carcinomas	50%
Penile carcinomas	30-50%
Anal and perianal carcinomas	60-94%

Table 1. Percentage of HPV detection in carcinomas of the anogenital region other than cervical carcinoma (Munoz et al., 2006; zur Hausen, 2009).

The most common viral types detected in cervical carcinomas include HPV 16, 18, 45, 31, 33, 52, 58, and 35 (Clifford et al., 2003; Munoz et al., 2003). The fraction of squamous cell carcinomas or adenocarcinomas attributable to HPV16 and HPV18, which comprise the two most common types, is 70% and 86%, respectively. The paradox is that, although infection with oncogenic types of HPV is very common, malignancy is a rare outcome. This difference in incidence between infection and cancer development reveals the significance of complex interactions between viral, environmental and host-related factors (Frazer, 2009; Moscicki et al., 2006; Snijders et al., 2006; Whiteside et al., 2008; zur Hausen, 2008). Viral persistence is an important determinant in this sequence of events, while immune status, viral integration into the host DNA, and infection with multiple HPV genotypes have significant roles. These multiple interactions are reflected in the long interval between infection and invasive carcinoma detection, often spanning a period of 15 to 25 years (zur Hausen 2008). Other factors that may modify the risk for HPV DNA-positive women include smoking, the use of oral contraceptives, and previous exposure to other sexually transmitted diseases (Bosch et al., 2006; Collins et al., 2010; Luie et al., 2011; Munoz et al., 2006).

In recent years a large number of scientific studies have resulted in the introduction of effective vaccines, which are expected to diminish the incidence of HPV-related carcinomas of the uterine cervix and other organs (Bogaardts et al., 2011; Frazer, 2009; Stanley, 2010; The FUTURE I/II Study Group, 2010; zur Hausen, 2008). Moreover, they are expected to reduce the incidence of intraepithelial HPV-related lesions. A large number of the latter are caused by non-carcinogenic HPV types and do not constitute precancerous lesions, but still may be

the cause of significant anxiety and distress for the patients. Furthermore, in rare instances they can give rise to life-threatening conditions, like recurrent respiratory papillomatosis.

Another main result of human papilloma virus research was the introduction in clinical practice of new diagnostic techniques (Cuzick et al., 2006; Gravitt et al., 2008; Poljak & Kocjan, 2010; Snijders et al., 2010). These allow for a more precise evaluation of the following: a) the presence of HPV in biologic specimens and the viral type present, b) the viral load, and c) the presence of an HPV-associated lesion demanding further therapeutic measures in cytological or biopsy material.

Finally, an important aspect of human papilloma virus research is the fact that the complex interactions between HPV oncoproteins and their multiple cellular targets offer to investigators the opportunity to study important cellular pathways related to the carcinogenic process in general.

1.2 Interactions between HPV oncoproteins and cellular pathways

High-risk mucosal HPVs encode three transforming proteins: E5, E6 and E7. Their multiple biological activities have been extensively studied in the last few decades; however, several aspects remain to be elucidated (McLaughlin-Drubin M & Münger K, 2009a).

HPV E5 is able to transform mouse fibroblasts and keratinocytes in culture (Straight et al., 1993). It is believed to contribute to early stages of carcinogenesis and works in concert with E6 and E7 (Talbert-Slagle & DiMaio, 2009; Hu et al., 2009). The latter proteins are necessary for the induction and maintenance of the transformed phenotype. They inhibit the function of tumor suppressors p53 and pRb, respectively, whereas their expression enables cells to bypass normal cell cycle checkpoints.

One of the main actions of HPV E7 proteins is the interaction with the retinoblastoma tumor suppressor protein, pRB, which controls S-phase entry through association with E2F transcription factor family members. They also interact with the related pocket proteins, p107 and p130. High-risk HPV E7 targets pRB for proteasomal degradation, while low-risk HPV E7 binds pRB with lower efficiency (approximately 10-fold lower) than HR- HPV E7 (McLaughlin-Drubin M & Münger K, 2009a; Munger et al., 1991). E7 proteins cause aberrant activation of cdk2 (cyclin dependent kinase 2), which is associated with cyclins E and A, as well as cdk inhibitors, mainly $p21^{CIP1}$ and $p27^{KIP1}$. E7 expression results in dysregulated expression of cyclins E and A (McLaughlin-Drubin M & Münger K, 2009b; Zerfass et al., 1995). It also results in retaining differentiating keratinocytes in a DNA synthesis competent state.

High-risk HPV E6 proteins target p53 for proteasomal degradation through association with the cellular ubiquitin ligase E6AP (McLaughlin-Drubin M & Münger K, 2009b; Scheffner et al., 1990). Low-risk HPV E6 proteins can also associate with E6AP; however, high-risk HPV proteins target p53 for ubiquitination.

Furthermore, HR-HPV E6 and E7 proteins cooperate to generate mitotic defects and aneuploidy through induction of supernumerary centrosomes and multipolar mitoses in epithelial cells (Duensing et al., 2000), while genomic instability results in the addition of

molecular alterations. The detection of abnormal mitoses is a useful morphologic indicator of high-risk HPV-associated lesions (Crum et al. 1984).

Finally, integration of HPV genome into host chromosomes is an important event in cervical carcinogenesis (Hopman et al., 2006; Pett & Coleman, 2007), which occurs frequently during malignant progression and may result in dysregulation of E6/E7 expression due to disruption of E2, with associated loss of the inhibitory E2 action.

2. Immunohistochemical stains in the diagnosis of Squamous Intraepithelial Lesions (SIL)

Clinical management of preinvasive cervical disease consists of confirmation of SIL diagnosis by histopathological examination, followed by treatment or careful follow-up of certain lesions, according to the current guidelines. Histopathological diagnosis of CIN is based on well-defined criteria. However, in certain cases distinguishing both low- and high-grade lesions from their mimics may pose problems (Crum & Rose, 2006; Kostopoulou et al., 2001; Kurman et al., 1992), even to experienced gynecologic pathologists. The distinction of florid reactive changes, immature metaplastic patterns, and atrophic changes from HPV-induced alterations may cause difficulties. Attempts have been made to redefine the traditional criteria for lesion diagnosis, while other efforts aimed at the adoption of new, more objective methods, which might support the former (Bollmann et al., 2005; Cho et al., 2005; Guillaud et al., 2005; Prasad et al., 1994; Salvia et al., 2004; Scheurer et al., 2007). However, studies attempting to correlate HPV presence and replication to certain cytohistologic alterations are becoming less frequent and/or fruitful.

In recent years molecular studies have revealed several markers that might be of utility in the diagnosis of squamous intraepithelial lesions, including cellular proteins targeted directly by viral oncoproteins, and markers related to the cell cycle, which is disturbed by multiple actions of the virus, as summarized in the above paragraphs. The immunohistochemical stains that are currently in use in several laboratories worldwide, as well as some new promising markers are presented in the following text. The terms low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL) will be used interchangeably with CIN1 and CIN2/3, respectively.

2.1 p16

One extensively studied marker is p16 INK4A (hereafter referred to as p16), a cyclin-dependent kinase inhibitor. p16 decelerates the cell cycle and functions as a tumor suppressor, while having a role in cellular senescence. p16 affects pRb-mediated regulation of the G1/S transition (Lukas et al., 1995; Ohtani et al., 2004; Quelle et al., 1995; Serrano, 1997; Sano et al., 1998).

The expression of p16 is altered in several human tumors by deletions, mutations, or methylation (Cohen & Geradts, 1997; Nakashima et al., 1999; O'Neill & McCluggage, 2006; Ruas & Peters 1998) and has also been altered in cervical carcinoma cases. However, increased expression is often observed in HPV-related intraepithelial lesions and this is mainly attributed to the presence of a feedback loop, which depends on the status of

retinoblastoma protein (pRb) and the potential of high-risk HPV E7 protein to inactivate the latter (Lukas et al., 1995; Giarre et al., 2001; McLaughlin-Drubin & Münger, 2009b). Correlation has been reported between HR-HPV oncogene expression and high scores of p16 positivity (Andersson et al., 2006), and enhancement of p16 RNA level has been observed in vitro after immortalization by high-risk HPV types (Nakao et al., 1997). Despite the presence of high levels of p16 in SILs, its suppressor function is not normally exerted.

Several groups of investigators have examined immunohistochemically the expression of p16 in cervical squamous intraepithelial lesions (reviewed by Kostopoulou et al., 2011) and its possible correlation with HR-HPV types and/or lesion "progression". Indeed, p16 is one of the best studied markers in gynaecologic pathology. However, percentages of immunohistochemical positivity vary among different studies, as presented in Table 2. In the latter, studies published in the last ten years and including more than 100 cases of squamous intraepithelial lesions in histopathologic specimens are summarized, and the reported percentages of p16 immunopositivity are presented, together with the criteria and the antibodies used by the authors. Importantly, different criteria have been used for p16 immunoreactivity evaluation, with some authors focusing only on diffuse immunopositivity, some reporting any type of immunostaining, and others reporting nuclear and cytoplasmic staining separately. It should be also noted that some authors interpret focal positivity as a false-positive reaction. Positivity in the studies presented below varied from 5.6% to 100% for low-grade lesions and from 45.2% to 100% for high-grade lesions (Table 2). The percentage of immunopositivity observed in non-neoplastic epithelia also varied between 0% and 32.7%.

In a recent study (Kostopoulou et al., 2011) the two basic patterns of immunoreactivity, that is focal and diffuse, were further subdivided into groups as following: Focal positivity was subdivided into cases with occasional positive cells, dispersed or in small groups, observed mainly in the lower epithelial layers, and cases with occasional positive cells, dispersed or in small groups, commonly above the parabasal layer. Diffuse positivity (Figure 1) in the horizontal plane involved either all epithelial layers, or only the basal, parabasal and intermediate layers, without extending to the upper third of the epithelium. In HSIL, only diffuse positivity was encountered, observed in 24/25 cases (96%). In LSIL 41/55 cases (74.5%) showed some type of positivity, most commonly focal/sporadic (Figures 2 and 3). Interestingly, three out of eight LSILs showing diffuse immunoreactivity were characterized by markedly increased nuclear dimensions in the upper epithelial layers in comparison to other lesions characterized as low-grade. Another interesting finding of the above study was the different HPV type distribution observed between the two patterns of sporadic/focal positivity, involving lower *vs* intermediate/upper epithelial layers, and probably reflecting an earlier sporadic expression of E7 in certain lesions (Kostopoulou et al., 2011). The percentage of high-risk or probable high-risk HPV associated LSILs positive for p16 was 71.4% (25/35). This was not significantly different from immunopositivity observed in low-risk HPV associated lesions. Moreover, study of the pertinent literature revealed that a significant percentage of LSILs testing positive for HR-HPV by PCR or HC2 does not exhibit any p16 immunopositivity. The percentage of p16 positivity reported for HR-HPV positive LSILs varied from 32.4% to 94.4% (Ishikawa et al. 2006; Kalof et al., 2005; Kostopoulou et al., 2011).

Reference	Number of SILs examined	LSIL positivity	HSIL positivity	Non-neoplastic epithelia	Evaluation of staining	Antibody used in the study
Agoff et al., 2003	269	56.6%	84.5%	11.5%	N and C ≥5% cells	E6H4 (MTM)
Branca et al., 2004	137	35%	81.2%	0%	N and/or C	Polyclonal (Abcam)
Negri et al., 2004	127	74.7%	100%	ND	N and C ≥5% cells in lower third	CINtec p16 Histology Kit (DakoCytomation)
Volgareva et, al. 2004	113	37.2%	45.2%	3.2%	N and/or C	E6H4 (MTM)
Wang et al., 2004	113	72%	94.7%	32.7%	Any reactivity	E6H4 (MTM)
Dray et al., 2005	104	74.1%	96.1%	7.0%	N and/or C	JC8 (Biocare Medical)
Murphy et al., 2005	117	100%	98.7%	0%	N or C	p16 (Pharmingen)
Ishikawa et al., 2006	141	24.5%	87.5%	0%	Moderate and strong	E6H4 (MTM)
Focchi et al., 2007	153	90.9%	100%	7.9%	C and N ≥5% cells	Ab7 16PO7 (Neomarkers)
Hariri & Oster, 2007	140	71.4%	100%	6%	Continuous basal and parabasal	p16 Histology Kit (Dako)
Van Niekerk et al., 2007	184	57.1%	96.9%	22.9%	N and C ≥5% cells in each layer	E6H4 (DakoCytomation)
Godoy et al. 2008	115	50%	96.2%	0%	C and N	CINtec p16 Kit (Dako)
Dijkstra et al., 2010	406	5.6%	96.7%	ND	Diffuse, >1/3 of epithelium	Ab-4, 16P04 (Lab Vision)
Tan et al., 2010	129	26.7%	79.7%	0%	N and C ≥5% cells	p16 (NeoMarkers)

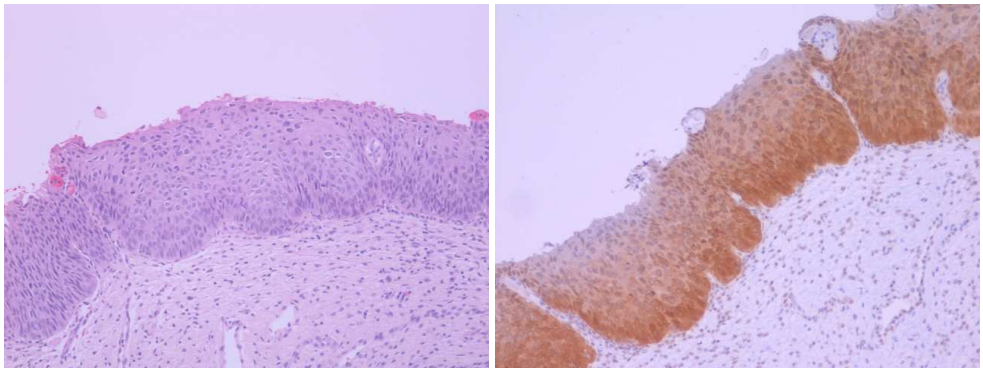
LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; N: nuclear; C: cytoplasmic; ND: no data

[a] Only studies including more than 100 cases of squamous intraepithelial lesions in histopathologic specimens and published in the last ten years are presented.

Table 2. p16 immunopositivity in low- and high- grade squamous intraepithelial lesions reported in the literature[a]

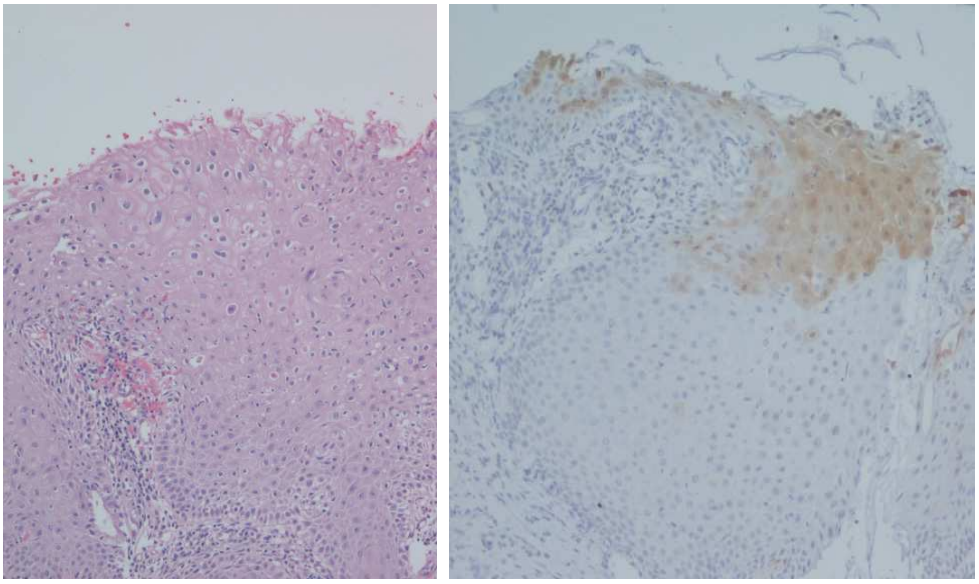
The results of the above studies point towards the use of p16 immunostain in conjunction with histopathologic evaluation. Addition of a consecutive p16-stained slide to the HE-stained slides has been shown to improve significantly interobserver agreement for both punch and cone biopsies (Bergeron et al., 2010; Dijkstra et al., 2010; Horn et al., 2008), and to help in the identification of occult lesions (Ordi et al., 2008). The differential diagnosis from non-neoplastic alterations can be facilitated, especially in conjunction with other immunostains, as presented below. Moreover, lesion grading can be faster, especially

concerning aggressive-appearing low-grade lesions, which otherwise might be upgraded (Dijkstra et al., 2010). Awareness of the different patterns of immunoreactivity might allow for a most proper use in certain clinicopathological settings. However, significant variability remains in the reported percentage of cases that stain positively for p16 and several unresolved technical issues remain, underlining the need for standardization of sample preparation and evaluation protocols (Mulvany et al., 2008; Tsoumpou et al., 2009).



(a) (b)

Fig. 1. (a,b). High-grade squamous intraepithelial lesion (HSIL-CIN2): (a) Hematoxylin and eosin staining, (b) p16 immunostain showing diffuse positivity.

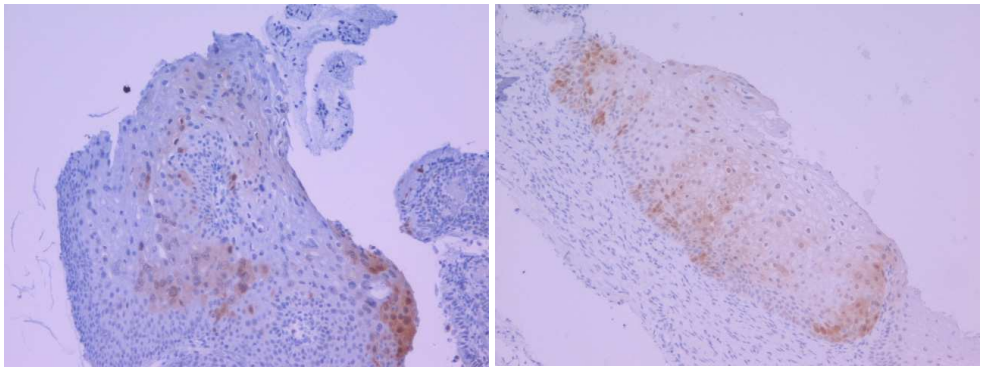


(a) (b)

Fig. 2. (a,b). Low-grade squamous intraepithelial lesion: (a) Hematoxylin and eosin staining, (b) p16 immunostain showing focal positivity.

It is of note that: (a) in several studies, especially with increasing number of cases, there often appears a small group of HSILs that do not show any immunoreactivity, and (b) a significant percentage of LSILs associated with HR-HPV, as detected by PCR or HC2, does not exhibit p16 immunopositivity (Kostopoulou et al., 2011). The above observations lead to the conclusion that a negative or equivocal p16 immunostain should be carefully evaluated in conjunction with the histopathologic findings and should not be used as the main criterion for diagnosis. However, p16 may also be of use in evaluating cauterized cervical resection margins, since the positive staining pattern of HGSIL is not affected by diathermy in LLETZ biopsies (Dray et al., 2005).

Finally, another aspect of p16 immunostaining is the possibility of correlation with lesion "progression". It has been suggested that certain phases of a given HR-HPV-associated neoplastic process may have different indices of p16 expression (Keating et al., 2001). Although the detailed examination of this subject is not included in the aim of the present text, it should be mentioned that in an interesting study by Hariri and Oster (2007) 25/26 low-grade lesions with negative p16 staining (concerning diffuse staining) and a minimum follow-up period of five years had a benign or normal outcome, revealing a negative predictive value of p16 in predicting the outcome of CIN 1 cases as high as 96%. In a study including conization specimens with coexisting CIN1 and CIN3 areas, all CIN1 were p16 positive (Negri et al., 2008), while p16 staining did not predict persistence or clearance of HR-HPV after treatment for CIN in a study by Branca et al. (2004).



(a) (b)

Fig. 3. (a,b). Common patterns of p16 positivity in low-grade lesions

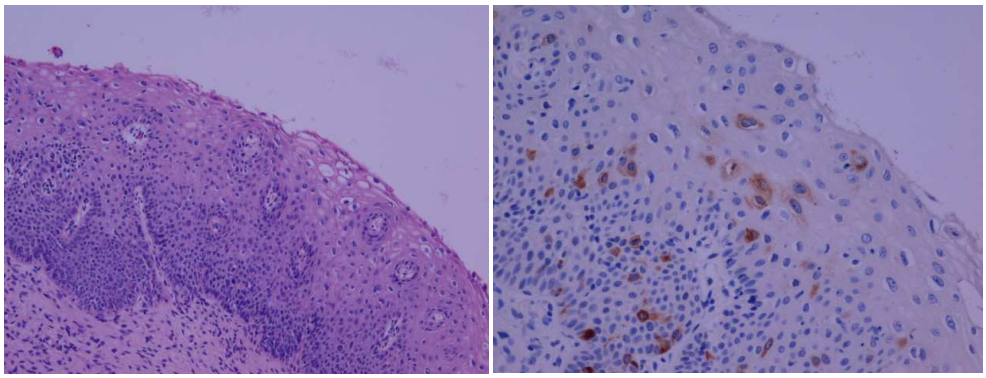
2.2 Cyclins

Cyclins have been reported to be of help in the evaluation of cervical biopsies. Cyclin E is uncommonly expressed in epithelia not infected by HPV and its conspicuous immunopositivity may facilitate the recognition of SIL (Keating et al., 2001). In addition, cyclin B1 immunoreactivity above the basal/parabasal cells correlates significantly with HPV detection and could be a marker of HPV presence (Kostopoulou et al., 2008a). Cyclins D and A have been also studied as possible markers of HPV-related lesions.

2.2.1 Cyclin B1

It has been reported that E6/E7 oncoproteins of HPV type 18 induced changes in the expression of cell cycle regulatory proteins very early and before immortalization (Pei, 1996). Significantly increased expression was noted for cyclin B and its transcriptional activation was documented. In 2000, Southern et al. demonstrated increased cyclin B1 expression in HGSILs. In their study cyclin B protein was up-regulated and persisted into the upper epithelial layers in parallel with cyclin A expression in high-grade squamous intraepithelial lesions.

In a study performed in our laboratory cyclin B1 immunostaining above the basal/parabasal layers was observed in all cases of HSIL (100%), most often involving the superficial layers as well (Kostopoulou et al., 2008a). Furthermore, increased cyclin B1 immunopositivity was observed in 51/52 low-grade lesions (98.07%) (Figure 4), and in seven of 15 biopsies (46.6%) characterized as atypia of unknown significance (AUS). Six of these seven cases tested HPV-positive by PCR.



(a) (b)

Fig. 4. (a,b). Low grade squamous intraepithelial lesion: (a) Hematoxylin and eosin staining, (b) Cyclin B1 immunostain, showing sporadic positivity in mature squamous polygonal cells above the basal layers.

The essential feature of the staining pattern observed in low-grade lesions and AUS cases in the above study consisted of sporadic cyclin B1 staining in mature squamous polygonal cells often just above the basal layers, with slight differences between flat and elevated lesions. This pattern of immunoreactivity was seen in 52 of 55 cases with HPV infection detected by PCR, whereas it was seen in only 5 cases without PCR-proven HPV infection. In 4 of the latter cases, however, p16 immunopositivity was detected, suggesting that HPV could be present though not detected by PCR.

The pattern of immunoreactivity observed in low-grade lesions and AUS cases could be perceived as cytoplasmic accumulation or retention of cyclin B1 in suprabasal squamous cells. Several mechanisms could be related to this reaction (Kostopoulou et al., 2008a), while this pattern might reflect early events in the inhibition of G2-to-M transition, a well-known phenomenon during HPV infection in vitro. The possibility was suggested that these cyclin

B1-positive cells could be viewed as a type of ‘‘prekoilocytes”, whose eventual progression to koilocytes would depend on several parameters related to the intricacies of HPV infection.

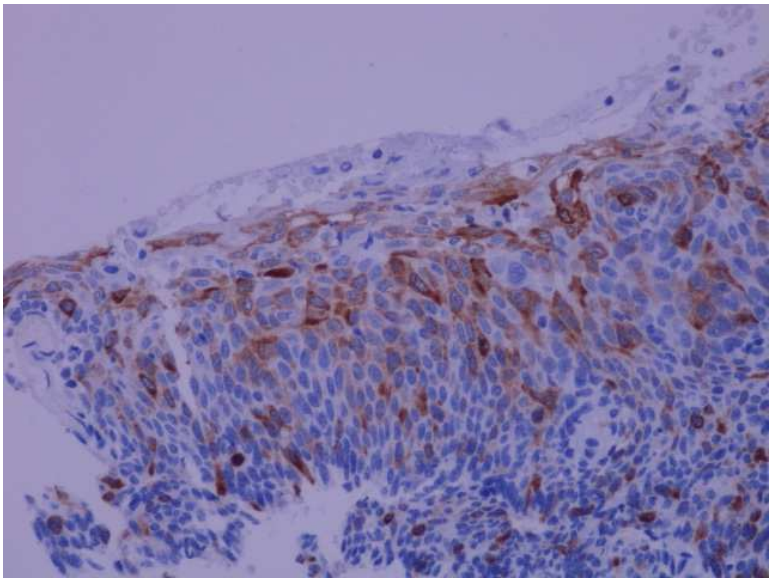


Fig. 5. Cyclin B1 positivity in an HSIL.

In conclusion, cyclin B1 positivity above the basal/parabasal layers correlates significantly with HPV detection and could be a marker of HPV presence. Thus, it might constitute a helpful finding in difficult to diagnose cases. Immunopositivity in a specimen showing non-diagnostic atypia should prompt reevaluation and/or HPV testing, as it is likely that the case could represent a genuine low-grade intraepithelial lesion.

2.2.2 Cyclin E

Cyclin E, another important cell cycle regulator, which promotes G1 transition, has been reported to exhibit increased expression in squamous intraepithelial lesions and invasive cervical carcinomas, although the exact mechanisms are not clear (Keating et al., 2001).

In a study by Keating et al., (2001) moderate to strong immunopositivity for cyclin E was observed in 92.6% and in 91.6% of low-grade and high-grade intraepithelial lesions, respectively, being positive in 38/41 HR-HPV positive cases. Furthermore, in a group of nondiagnostic squamous atypias cyclin E positivity was associated with HPV positivity.

In a study by Bahnassy et al. (2007), cyclin E staining increased from CIN1 to invasive carcinoma (16.7% to 88.4%, respectively), while gene amplification was detected in 11.1% of CIN1 cases and in 88.4% of carcinoma cases.

In conclusion, although cyclin E staining is not useful in the distinction of low-grade from high-grade lesions, it could be used to discriminate reactive from neoplastic epithelium (Crum & Rose, 2006), especially in conjunction with other markers, as discussed in other

parts of the present text. As is the case with the other immunostains examined in this text, standardization of staining and evaluation protocols are important for the appropriate application of these markers in certain diagnostic dilemmas.

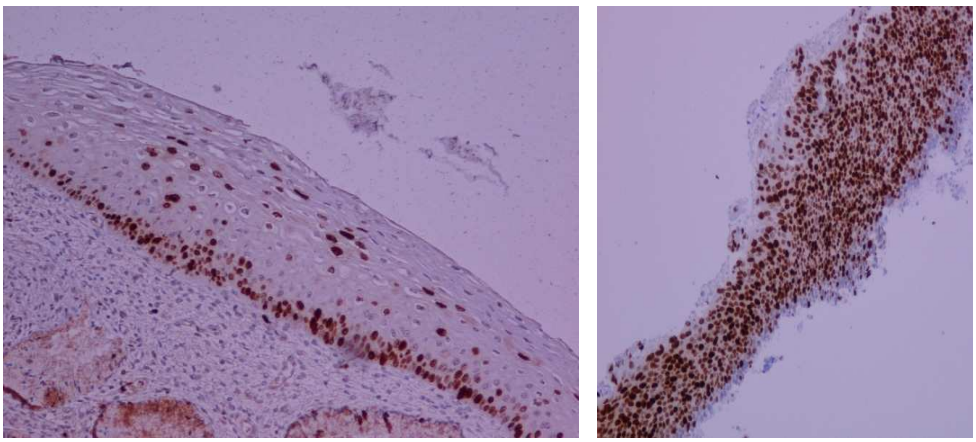
2.3 Other proliferation/cell cycle markers

2.3.1 Ki-67

Ki-67, an antigen expressed in the nuclei of proliferating cells, has also been studied as an indicator of CIN. Ki-67 is expressed in the nucleus during the whole cell cycle, except for the G0 and G1 early phases. Although positivity is observed under normal conditions in the lower compartments of the multilayered squamous epithelium, staining of the middle and upper layers is indicative of an intraepithelial lesion (Figure 6).

Immunopositivity for Ki-67 increases as a function of increasing lesion grade (Arafa et2008; *Conesa-Zamora* et al., 2009; Carreras et al., 2007; Keating et al., 2001; Mimica et al., 2010; Pinto et al., 2008), but immunostains should be interpreted with caution, since reactive and inflammatory lesions may result in increased epithelial proliferation. It is well-known to pathologists that reactive and reparative changes may pose a problem in the examination of proliferation markers and in the case of Ki-67 immunostaining positive nuclei may extend through most of the epithelium. However, Ki-67 immunostaining can be used as an adjunct to other markers, as already discussed.

It should be noted that Ki-67 immunohistochemical stain may be especially helpful in differentiating atrophic epithelial changes from high-grade lesions (Crum & Rose, 2006).



(a) (b)

Fig. 6. (a,b). Ki-67positivity in (a) a low- and (b) a high-grade intraepithelial lesion.

2.3.2 Aberrant S-phase

Two relatively new biomarkers include the minichromosome maintenance protein 2 (MCM2) and DNA topoisomerase IIa (TOP2A) (Pinto et al., 2008). These two proteins have a significant role in the regulation of DNA replication during S-phase. They are overexpressed

when S-phase induction is aberrant and have been shown to be overexpressed in CINs and cervical carcinomas (Badr et al., 2008; Pinto et al., 2008; Shi et al., 2007). TOP2A is a nuclear enzyme that regulates the enzymatic unlinking of DNA strands during chromosome replication. MCM2 functions also during DNA replication by loading the pre-replication complex onto DNA and unwinding the latter through helicase activity to permit synthesis. ProEx C (Tambouret et al., 2008) is a recently developed immunohistochemical assay that targets these two proteins and appears to be efficient in distinguishing reactive epithelial changes from squamous lesions, alone or in conjunction with p16.

According to Shi et al. (2007), ProEXC is a better marker than p16 for the detection of LSILs, showing positivity in 94% of the cases in a series of 34 LSILs. In a study by Badr et al. (2008) strong positive staining for ProEx C involving the lower and upper halves of the epithelium was observed in 92% of high-grade squamous intraepithelial lesions. Condylomas and CIN I showed greater variability in patterns of staining, with immunopositivity extending into the upper half of the epithelium in 48% of cases.

Pinto et al. (2008) included in their study cases with the differential diagnosis of HSIL *vs* reactive epithelial changes. ProEx C showed 87% sensitivity and 71% specificity for SIL in biopsy material. The authors reported a larger number of cells stained by ProEx C in comparison to MiB-1 in both HSIL and LSIL cases. In addition, the combination of p16 and ProEx C predicted more NoSIL (including normal, reactive, and/or atrophic epithelia) than p16 and MiB-1 (61% vs 43%). These observations suggested that ProEx C could be more useful in the distinction of reactive epithelial changes from SILs than MiB-1, providing a lower false positive rate relative to the latter.

In a study by Sanati et al. (2010) sensitivity, specificity, positive and negative predictive value of ProExC in distinguishing high-grade squamous intraepithelial lesion from squamous metaplasia were 89%, 100%, 100%, and 82%, respectively. In a recent study by Guo et al., (2011) diffuse positivity for ProExC significantly increased from benign cervix/CIN 1 to CIN 2 or 3/carcinoma, while the highest specificity for CIN 2+ and CIN3+ (100% and 93%, respectively) was achieved when immunostaining was positive for both ProExC and p16, suggesting that it is advantageous to use these two markers together in order to distinguish high-grade lesions from their mimics.

Walts and Bose (2009) suggested as cost saving strategy the use of two markers initially, p16 and ProExC, followed by Ki-67 immunostaining in discordant cases. According to the above authors, performing the two above stains initially and adding Ki-67 only when p16 and ProExC yield discordant results provided the same diagnostic accuracy while reducing the cost, since only one third of the cases required performance of the third stain.

2.4 Other markers and applications

In the present text an effort has been made to cover the immunohistochemical markers, which are currently most useful from a diagnostic point of view, and have been evaluated in several studies and laboratories.

In addition to the above biomarkers, which are in use in many pathology departments worldwide, a large number of other markers have been examined for their potential utility in the diagnosis and/or prognosis of cervical precursor lesions and in resolving problematic

cases (Galgano et al., 2010; Khan et al., 2008; Kostopoulou et al., 2008b). The results of these studies have been described in detail in the pertinent literature. In addition, image analysis methods have been used in an attempt to bring more objectivity to the interpretation of biopsy specimens. Furthermore, although the detection of SILs in cytology material and the evaluation of screening strategies are beyond the scope of the present text, it should be mentioned that the contribution of the above markers is important in this context, as presented in brief in the following (Carozzi et al., 2008; Depuydt et al., 2011; Tsoumpou et al., 2009).

2.4.1 L1 capsid protein

One recently studied marker, which has been examined repeatedly in cytologic material, is L1. Nuclear positivity for HPV L1 capsid protein, the major structural protein of human papillomavirus, is mainly observed in productive lesions and is gradually lost in high grade lesions and carcinomas.

It has been suggested that combined L1/p16 immunostaining may be helpful for clinical management, especially in cases in which the grade of the lesion is difficult to assess (Negri et al., 2008).

In a study by Galgano et al. (2010), this protein, which should be highly correlated with a productive viral infection, was neither sensitive nor specific for any group of cervical neoplasia in biopsy material. This was attributed to the complexity of the temporal evolution of the HPV virion production which may be quite transient. It is interesting that L1 positive cases with a negative consensus diagnosis in this study had commonly at least 1 reviewer diagnosis of CIN1, revealing once again the difficulties in the distinction of SIL *vs* negative for SIL and the importance of a panel of immunostains in this specific context.

2.4.2 In situ hybridization techniques

Detection of papillomavirus nucleic acids is currently performed by methods that can be broadly subdivided into methods based on target amplification and those based on signal amplification (Snijders et al., 2010). In addition to several existing liquid phase techniques, in situ hybridization (ISH) methods have been developed for cytological and histological specimens. Both fluorescent detection and coloured substrate deposition followed by bright-field microscopy can be used, and can be combined with tyramide signal amplification. ISH assays can also be automated along the same lines as immunohistochemistry. Finally, except for HPV nucleic acids, other applications of in situ hybridization include the detection of amplification of the gene coding for the telomerase RNA component (TERC) at 3q26 (Hopman et al., 2006; Zheng et al., 2010).

Issues concerning sensitivity of the above techniques in comparison to PCR have been repeatedly raised. However, ISH techniques are becoming increasingly sensitive and can now detect low copy numbers of HPV DNA (Kelesidis et al., 2011; Montag et al., 2011). In addition, their important contribution to HPV research is the fact that they allow concurrent morphological evaluation of the areas examined, mainly in the case of histological specimens. Furthermore, the signal patterns observed in HPV ISH have been reported to be associated with the physical status of viral DNA in the cells examined, that is episomal or integrated. Specifically, the punctate pattern of positivity has been linked to the presence of

integrated viral forms in the host genome (Cooper et al., 1991; Evans et al., 2002; Hopman et al., 2005).

In a study by Guo et al. (2008) ISH and PCR had fair to good agreement in detecting HPV DNA across CIN categories, but ISH detected significantly fewer HPV-positive cases in carcinomas than PCR did, probably as a result of lower copy numbers of episomal as compared to integrated HPV DNA in the latter. In addition, although the pure punctate pattern of HPV indicated a high level of viral integration, the level of HPV integration could not be accurately determined in cases with mixed signal patterns, probably due to a variation in the percentage of the two patterns in these cases. Recently, Ho et al. (2011) reported a punctate pattern in 8.7% of CIN1 lesions *vs* 34.0% of CIN3 lesions in cytology material, while Alameda et al. (2011) reported a correlation of the punctate pattern with lesion persistence in cytology specimens.

According to Kong et al. (2007), in cases of atypical squamous metaplasia, p16 reactivity (focal strong and diffuse strong) was significantly more sensitive than ISH in correlating with the presence of human papillomavirus as detected by polymerase chain reaction. In a more recent study by Kelesidis et al. (2011), ISH exhibited a sensitivity of 89.5% for the detection of CIN2+ lesions, while PCR showed sensitivity of 94.7% for these lesions. A percentage of ISH-positive cases was not detected by PCR (performed on liquid-based sample media), emphasizing the technical problems and limitations of the techniques.

Voss et al. (2009) compared a fluorescence in situ hybridization (FISH) HR-HPV assay to Hybrid Capture 2 (HC2) and polymerase chain reaction (PCR) for the detection of HR-HPV subtypes in cervical cytology specimens. FISH was concordant with HC2 and PCR in 85% and 82% of the specimens, respectively, while HC2 and PCR were concordant in 84% of the specimens.

It is apparent from the above results that the applications of HPV ISH are partly dependent on the sensitivity of the assay and its sufficiency to carry a high negative predictive value (Crum & Rose 2006). This is especially important if clinical decisions are based on a negative result. However, ISH represents a useful tool for ancillary molecular HPV testing in cervical specimens, and may be important in certain clinicopathologic situations.

2.4.3 Applications in cytology

The preceding text focused mainly on the application of immunohistochemistry in SIL diagnosis in histopathology specimens. However, several of the above markers have been applied in cytopathology material, as presented in brief in the following paragraphs. The introduction of liquid-based techniques, which has been one of the most important advances in this field, has facilitated relevant applications.

The most studied marker in cytology is p16. Positivity has been observed in 10%-86% of LSIL and in 42%-100% of HSIL, as reviewed by Tsoumpou et al. (2009). The lack of general consensus regarding threshold values for p16 positivity is especially important in cervical cytology specimens. Several authors have used both quantitative and qualitative criteria, evaluating the number of positive cells as well as cell morphology, recognizing the fact that p16 overexpression may be often detected in nondysplastic cells. In the contrary, other investigators used only quantitative criteria.

It has been suggested that p16 immunocytochemical testing can be used as a reflex test in conjunction with liquid-based cytology following a cytologic result of ASC-US or LSIL, or be used on destained conventional or liquid-based cytology specimens (Denton et al., 2010). p16 in conjunction with Ki-67 provide high sensitivity for the detection of CIN2+ lesions (Schmidt et al., 2011; Yu et al., 2010).

The prognostic utility of L1 immunocytochemistry, especially in association with p16 in cytology, has been reported by several authors (Griesser et al., 2004; Sarmadi et al., 2011; Yoshida et al., 2008).

The use of HPV in situ hybridization has already been discussed in the previous section. It is of note that, in a recent study, prior knowledge of HPV status resulted in significantly higher detection rate of CIN2+ in cytology specimens compared to screening blinded to HPV status, with limited loss of specificity (Benoy et al., 2011). This raises several important questions, although more research is needed to study the significance of this type of knowledge provided prior to cytological reading.

3. Conclusion

Although histopathology remains the ''gold standard'' for the diagnosis of SIL, both low- and high-grade, certain biomarkers have emerged as helpful adjuncts. Their combined use may assist in the histopathologic classification of preinvasive lesions and facilitate the distinction from non-HPV induced alterations. It is clear from the above that the diagnosis of a squamous intraepithelial lesion in a diagnostically challenging case cannot at present be based solely on any particular marker, but rather on a combination of markers with careful morphological evaluation, the latter comprising the most important part of the diagnostic procedure. Standardization of protocols and familiarity with the patterns of immunostaining, especially in nonneoplastic cervical tissue, are important requirements for the proper use of the above markers. Awareness of the strengths and limitations of each particular technique cannot be overemphasized. In addition, the performance of several markers and methods in the detection of lesions related to HPV types other than those addressed by the current vaccines remains to be carefully evaluated.

4. References

Agoff, SN., Lin, P., Morihara, J., Mao, C., Kiviat, N.B. & Koutsky, L.A. (2003). p $^{16(INK4a)}$ expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. *Modern Pathology,* Vol.16, pp.665-673

Alameda, F., Mariñoso, M.L., Bellosillo, B., Muset, M., Pairet, S., Soler, I., Romero, E., Larrazabal, F., Carreras, R. & Serrano, S. (2011). Detection of HPV by in situ hybridization in thin-layer (ThinPrep) cervicovaginal samples. *Tumour Biology,* Vol.32, No.3, pp.603-609, Epub 2011 Feb 8

Andersson, S., Hansson, B., Norman, I., Gaberi, V., Mints, M., Hjerpe, A., et al. (2006). Expression of E6/E7 mRNA from 'high risk' human papillomavirus in relation to

CIN grade, viral load and p16INK4a. *International Journal of Oncology,* Vol. 29, No.3, pp. 705-711

Arafa, M., Boniver, J & Delvenne P. (2008). Detection of HPV-induced cervical (pre) neoplastic lesions: a tissue microarray (TMA) study. *Applied Immunohistochemistry and Molecular Morphology*, Vol.16, No.5, pp.422-432

Badr, R.E., Walts, A.E., Chung, F. & Bose, S. (2008). BD ProEx C: a sensitive and specific marker of HPV-associated squamous lesions of the cervix. *American Journal of Surgical Pathology,* Vol.32, pp.899-906

Bahnassy, A., Zekri, A., Saleh, M., Lotayef, M., Moneir, M. & Shawki, O. (2007). The possible role of cell cycle regulators in multistep process of HPV-associated cervical carcinoma. *BMC Clinical Pathology,* Vol.7, pp.4

Benoy, I., Vanden Broeck, D., Ruymbeke, M., Sahebali, S., Arbyn, M., Bogers, J., Temmerman, M. & Depuydt, C. Prior knowledge of HPV status improves detection of CIN2+ by cytology screening. *American Journal of Obstetrics and Gynecology,* in press.

Bergeron, C., Ordi, J., Schmidt, D., Trunk, M., Keller, T. & Ridder, R., for the European CINtec Histology Study Group. (2010). Conjunctive p16INK4a Testing Significantly Increases Accuracy in Diagnosing High-Grade Cervical Intraepithelial Neoplasia. *American Journal of Clinical Pathology,* Vol.133, pp.395-406

Bergonzini, V., Salata, C., Calistri, A., Parolin, C. & Palu, G. (2010). View and review on viral oncology research. *Infectious Agents and Cancer,* Vol.5:, pp.11

Bogaards, J.A., Coupé, V.M., Xiridou, M., Meijer, C.J., Wallinga, J. & Berkhof, J. (2011). Long-term Impact of Human Papillomavirus Vaccination on Infection Rates, Cervical Abnormalities, and Cancer Incidence. *Epidemiology,* Vol.22, No.4, pp.505-515

Bollmann, M., Bankfalvi, A., Trosic, A., Speich, N., Schmitt, C. & Bollmann, R. (2005). Can we detect cervical human papillomavirus (HPV) infection by cytomorphology alone? Diagnostic value of non-classic cytological signs of HPV effect in minimally abnormal Pap tests. *Cytopathology,* Vol.16, pp.13–21

Bosch, F.X., Lorincz, A., Munoz, N., Meijer, C.J. & Shah, K.V. (2002). The causal relation between human papillomavirus and cervical cancer. *Journal of Clinical Pathology.*, Vol.55, pp.244-265

Bosch, F.X., Qiao, Y. & Castellsague, X. (2006). The epidemiology of human papillomavirus infection and its association with cervical cancer. *International Journal of Gynecology and Obstetrics*, Vol.94(Supplement 1), pp.S8---S21

Branca, M., Ciotti, M., Santini, D., Di Bonito, L., Giorgi, C., Benedetto, A., et al. (2004). $p16^{INK4a}$ expression is related to grade of CIN and high-risk human papillomavirus but does not predict virus clearance after conization or disease outcome. *International Journal of Gynecological Pathology,* Vol. 23, pp.354-365

Carozzi, F., Confortini, M., Dalla Palma, P., Del Mistro, A., Gillio-Tos, A., De Marco, L., Giorgi-Rossi, P., Pontenani, G., Rosso, S., Sani, C., Sintoni, C., Segnan, N., Zorzi, M., Cuzick, J., Rizzolo, R., Ronco, G.; New Technologies for Cervival Cancer Screening (NTCC) Working Group. (2008). Use of p16-INK4A overexpression to increase the specificity of human papillomavirus testing: a nested substudy of the NTCC randomised controlled trial. *Lancet Oncology,* Vol.9, No.10, pp.937-945, Epub 2008 Sep 8.

Carreras, R., Alameda, F., Mancebo, G., García-Moreno, P., Mariñoso, M.L., Costa, C., Fusté, P., Baró, T. & Serrano, S. (2007). A study of Ki-67, c-erbB2 and cyclin D-1 expression in CIN-I, CIN-III and squamous cell carcinoma of the cervix. *Histology and Histopathology,* Vol.22, No.6, pp.587-592

Cho, N.H., Kang, S., Hong, S., Jeong, G.B., Choi, I.W., Choi, H.J. & Choi, H.K. (2005). Multinucleation of koilocytes is in fact multilobation and is related to aberration of the G2 checkpoint. *Journal of Clinical Pathology,* Vol.58, pp.576–582

Clifford, G.M., Smith, J.S., Plummer, M., Munoz, N. & Franceschi, S. (2003). Human papillomavirus types in invasive cervical cancer worldwide: a metaanalysis. *British Journal of Cancer*, Vol.88, No.1, pp.63–73

Cohen, J.A. & Geradts, J. (1997). Loss of Rb and MTS1/CDKN2 (P16) expression in human sarcomas. *Human Pathology,* Vol.28, pp.893–898

Collins, S., Rollason, T., Young, L. & Woodman C. (2010). Cigarette smoking is an independent risk factor for cervical intraepithelial neoplasia in young women: A longitudinal study. *European Journal of Cancer*, Vol. 46, No.2, pp.405–411

Conesa-Zamora, P., Domenech-Peris, A., Orantes-Casado, F., Ortiz-Reina, S., Sahuquillo-Frias, L., Acosta-Ortega, J., Garcia-Solano, J. & Perez-Guillermo, M. (2009). Effect of Human Papillomavirus on Cell Cycle–Related Proteins p16, Ki-67, Cyclin D1, p53, and ProEx C in Precursor Lesions of Cervical Carcinoma. *American Journal of Clinical Pathology*, Vol.132, pp.378-390

Cooper, K., Herrington, C.S., Stickland, J.E., Evans, M.F. & McGee J.O.. (1991). Episomal and integrated human papillomavirus in cervical neoplasia shown by non-isotopic in situ hybridization. *Journal of Clinical Pathology,* Vol.44, pp.990–996

Crum, C.P., Ikenberg, H., Richart, R.M. & Gissmann, L. (1984). Human papillomavirus type 16 and early cervical neoplasia. *New England Journal of Medicine*, Vol.310, pp.880–883

Crum, C.P. Contemporary theories of cervical carcinogenesis: the virus, the host, and the stem cell. (2000). *Modern Pathology*, Vol.13, pp.243–251

Crum, C.P., Abbott, D.W. & Quade, B.J. (2003). Cervical cancer screening: from the Papanicolaou smear to the vaccine era. *Journal of Clinical Oncology*, Vol. 21(10 Suppl), pp.224–230

Crum, C.P. & Rose, P. (2006). Cervical squamous neoplasia. In: *Diagnostic Gynecologic and Obstetric Pathology.* C.P. Crum & K.R. Lee, (Eds.), 267-354, Elsevier

Cuzick, J., Mayrand, M., Ronco, G., Snijders, P. & Wardle, J. (2006). Chapter 10: New dimensions in cervical cancer screening. *Vaccine,* Vol.24(S3), pp.S90-97

Denton, K., Bergeron, C., Klement, P., Trunk, M., Keller, T. & Ridder, R., for the European CINtec Cytology Study Group. (2010). The Sensitivity and Specificity of p16INK4a Cytology vs HPV Testing for Detecting High-Grade Cervical Disease in the Triage of ASC-US and LSIL Pap Cytology Results. *American Journal of Clinical Pathology,* Vol.134, pp.12-21

Depuydt, C.E., Makar, A.P., Ruymbeke, M.J., Benoy, I.H., Vereecken, A.J. & Bogers, J.J. (2011). BD-ProExC as adjunct molecular marker for improved detection of CIN2+ after HPV primary screening. *Cancer Epidemiology, Biomarkers and Prevention*, Vol.20, No.4, pp.628-637, Epub 2011 Feb 4

Dijkstra, M.G., Heideman, D.A., de Roy, S.C., Rozendaal, L., Berkhof, J., van Krimpen, K., van Groningen, K., Snijders, P.J., Meijer, C.J. & van Kemenade, F.J. (2010).

p16(INK4a) immunostaining as an alternative to histology review for reliable grading of cervical intraepithelial lesions. *Journal of Clinical Pathology*, Vol.63(, No.11, pp.972-927, Epub 2010 Oct 5

Dray, M., Russell, P., Dalrymple, C., Wallman, N., Angus, G., Leong, A. et al. (2005). p16^{INK4a} as a complementary marker of high-grade intraepithelial lesions of the uterine cervix. I: Experience with squamous lesions in 189 consecutive cervical biopsies. *Pathology,* Vol. 37, No.2, pp.112–124

Duensing, S., Lee, L.Y., Duensing, A., Basile, J., Piboonniyom, S., Gonzalez, S., Crum, C.P. & Munger, K. (2000). The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proceedings of the National Academy of Sciences*, Vol.97, pp.10002–10007

Evans, M.F., Mount, S.L., Beatty, B.G. & Cooper, K. (2002). Biotinyltyramide-based in situ hybridization signal patterns distinguish human papillomavirus type and grade of cervical intraepithelial neoplasia. *Modern Pathology*,Vol.15, pp.1339–1347

Focchi, G., Silva, I., Nogueira-de-Souza, N., Dobo, C., Oshima, C. & Stavale, J. (2007). Immunohistochemical Expression of p16(INK4A) in Normal Uterine Cervix, Nonneoplastic Epithelial Lesions, and Low-grade Squamous Intraepithelial Lesions. *Journal of Lower Genital Tract Disease*, Vol.11, pp.98-104

Frazer, I. (2009). Interaction of human papillomaviruses with the host immune system: A well evolved relationship. *Virology*, Vol.384, pp.410–414

Fuste, V., del Pino, M., Perez, A., Garcia, A., Torne, A., Pahisa, J. & Ordi, J. (2010). Primary squamous cell carcinoma of the vagina: human papillomavirus detection, p16INK4A overexpression and clinicopathological correlations. *Histopathology*, Vol.57, pp.907–916

Giarrè, M., Caldeira, S., Malanchi, I., Ciccolini, F., Leão, M.J. & Tommasino, M. (2001). Induction of pRb degradation by the human papillomavirus type 16 E7 protein is essential to efficiently overcome p16INK4a-imposed G1 cell cycle arrest. *Journal of Virology*, Vol. 75, No.10, pp.4705-4712

Godoy, A., Mandelli, J., Oliveira, F., Calegari, S., Moura, L. & Serafini, E. (2008). p16INK4 expression in precursor lesions of squamous cell cervical cancer related to the presence of HPV-DNA. *Brazilian Journal of Medical and Biological Research*, Vol.41, pp.583-588

Gravitt, P.E., Coutlıe, F., Iftner, T., Sellors, J.W., Quint, W.G. & Wheeler, C.M. (2008). New technologies in cervical cancer screening. *Vaccine,* Vol.26, No.Suppl.10, pp.42-52

Griesser, H., Sander, H., Hilfrich, R., Moser, B. & Schenck U. (2004). Correlation of immunochemical detection of HPV L1 capsid protein in pap smears with regression of high-risk HPV positive mild/moderate dysplasia. *Analytical and Quantitative Cytology and Histology*, Vol.26, No.5, pp.241-245

Gross, G. & Pfister, H. (2004). Role of human papillomavirus in penile cancer, penile intraepithelial squamous cell neoplasias and in genital warts. *Medical Microbiology and Immunology*, Vol.193:, pp.35–44

Guillaud, M., Adler-Storthz, K., Malpica, A., Staerkel, G., Matisic, J., Van Niekirk, D., Cox, D., Poulin, N., Follen, M. & MacAulay, C. (2005). Subvisual chromatin changes in cervical epithelium measured by texture image analysis and correlated with HPV. *Gynecologic Oncology*, Vol.99, No.3suppl 1), pp.16–23

Guo, M., Baruch, A.C., Silva, E.G., Jan, Y.J., Lin, E., Sneige, N. & Deavers M.T. (2011). Efficacy of p16 and ProExC immunostaining in the detection of high-grade cervical intraepithelial neoplasia and cervical carcinoma. *American Journal of Clinical Pathology,* Vol.135, No.2, pp.212-220

Guo, M., Gong, Y., Deavers, M., Silva, E.G., Jan, Y.J., Cogdell, D.E., Luthra, R., Lin, E., Lai, H.C., Zhang, W. & Sneige, N. (2008). Evaluation of a commercialized in situ hybridization assay for detecting human papillomavirus DNA in tissue specimens from patients with cervical intraepithelial neoplasia and cervical carcinoma. *Journal of Clinical Microbiology,* Vol.46, No.1, pp.274-280, Epub 2007 Oct 31

Hariri, J. & Oster, A. (2007). The Negative Predictive Value of p16INK4a to Assess the Outcome of Cervical Intraepithelial Neoplasia 1 in the Uterine Cervix. *International Journal of Gynecological Pathology*, Vol.26, pp.223–228

Ho, C.M., Lee, B.H., Chang, S.F., Chien, T.Y., Huang S.H., Yan, C.C. & Cheng, W.F. (2011). Clinical significance of signal pattern of high-risk human papillomavirus using a novel fluorescence in situ hybridization assay in cervical cytology. *Clinical Microbiology and Infection,* Vol.17, No.3, pp.386-394

Hopman, A. H., Kamps, M. A., Smedts, F., Speel E. J., Herrington, C. S., & Ramaekers, F. C.. (2005). HPV in situ hybridization: impact of different protocols on the detection of integrated HPV. *International Journal of Cancer,* Vol.115, pp.419–428

Hopman, A.H.,Theelen, W., Hommelberg, P.P., Kamps, M.A., Herrington, C.S., Morrison, L.E., Speel, E.J., Smedts, F. & Ramaekers, F.C. (2006). Genomic integration of oncogenic HPV and gain of the human telomerase gene TERC at 3q26 are strongly associated events in the progression of uterine cervical dysplasia to invasive cancer. *Journal of Pathology,* Vol.210, No.4, pp.412-419

Horn, L., Reichert, A., Oster, A., Arndal, S., Trunk, M., Ridder, R., et al. (2008). Immunostaining for p16INK4a Used as a Conjunctive Tool Improves Interobserver Agreement of the Histologic Diagnosis of Cervical Intraepithelial Neoplasia. *American Journal of Surgical Pathology*, Vol. 32, pp.502–12

Hu, L., Plafker, K., Vorozhoko, V., Zuna, R., Hanigan, M., Gorbsky, G., Plafker, S., Angeletti, P. & Ceresa, B. (2009). Human Papillomavirus 16 E5 Induces Bi-Nucleated Cell Formation By Cell-Cell Fusion. *Virology,* Vol.384, No.1, pp.125–134

Insinga, R., Liaw, K., Johnson, L., & Madeleine, M. (2008). A Systematic Review of the Prevalence and Attribution of Human Papillomavirus Types Among Cervical, Vaginal and Vulvar Precancers and Cancers in the United States. *Cancer Epidemiology Biomarkers and Prevention*. Vol. 17, No.7, pp.1611–1622

Ishikawa, M., Fujii, T., Saito, M., Nindl, I., Ono, A., Kubushiro, K., et al. (2006). Overexpression of $p16^{INK4a}$ as an indicator for human papillomavirus oncogenic activity in cervical squamous neoplasia. *International Journal of Gynecological Cancer*, Vol.16, No.1, pp.347–353

Kalof, A.N., Evans, M.F., Simmons-Arnold, L., Beatty, B. & Kumarasen, C. (2005). p16INK4A immunoexpression and HPV in situ hybridization signal patterns: potential markers of high-grade cervical intraepithelial neoplasia. *American Journal of Surgical Pathology,* Vol.29, pp.674–679

Keating, J.T., Cviko, A., Riethdorf, S., Riethdorf, L., Quade, B.J., Sun, D., et al. (2001). Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human

papilloma virus-related cervical neoplasia. *American Journal of Surgical Pathology,* Vol. 25, pp.884–891

Kelesidis, T., Aish, L., Steller, M.A., Aish, I.S., Shen, J., Foukas, P., Panayiotides, J., Petrikkos, G., Karakitsos, P. & Tsiodras, S. (2011). Human Papillomavirus (HPV) Detection Using In Situ Hybridization in Histologic Samples: Correlations With Cytologic Changes and Polymerase Chain Reaction HPV Detection. *American Journal of Clinical Pathology,* Vol.136, No.1, pp.119-127

Khan, A.M. & Singer, A. (2008). Biomarkers in cervical precancer management: the new frontiers. *Future Oncology,* Vol.4, No.4, pp.515-524

Kong, C.S., Balzer, B.L., Troxell, M.L., Patterson, B.K. & Longacre, T.A. (2007). p16INK4A immunohistochemistry is superior to HPV in situ hybridization for the detection of high-risk HPV in atypical squamous metaplasia. *American Journal of Surgical Pathology,* Vol.31, No.1, pp.33-43

Kostopoulou, E., Keating, J.T. & Crum, C.P. (2001). Pathology. In: *American Cancer Society Atlas of Clinical Oncology. Cancer of the female lower genital tract.* P.J. Eifel, C. Levenback, (Eds), 9-36, BC Decker, Hamilton, London

Kostopoulou, E., Samara, M., Kollia, P., Zacharouli, K., Mademtzis, I., Daponte, A., Messinis, I.E. &, Koukoulis, G. (2008a). Correlation Between Cyclin B1 Immunostaining in Cervical Biopsies and HPV Detection by PCR. *Applied Immunohistochemistry and Molecular Morphology*, Vol.17, No.2, pp.115-120, Epub Oct 28; 2008a.

Kostopoulou, E., Samara, M., Kollia, P., Zacharouli, K.& Koukoulis, G. (2008b). INCENP, cyclin B1 and ERK1 immunopositivity in cervical biopsies. *Histopathology*, Vol.53, No.438Sp.Iss., pp.192-193

Kostopoulou, E., Samara, M., Kollia, P., Zacharouli, K., Mademtzis, I., Daponte, A., Messinis, I.E. & Koukoulis, G. (2011). Different patterns of p16 immunoreactivity in cervical biopsies: Correlation to lesion grade and HPV detection, with review of the literature. *European Journal of Gynaecological Oncology,* Vol.32, No.1, pp.54-61

Kurman, R., Norris, H. & Wilkinson, E. (1992). *Tumors of the cervix, vagina and vulva. A.F.I.P. Atlas of tumor Pathology, 3rd series.* A.F.I.P., Washington

Louie, K.S., Castellsague, X., de Sanjose, S., Herrero, R., Meijer, C.J., Shah, K., Munoz, N., Bosch, F.X.; for the International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. (2011). Smoking and Passive Smoking in Cervical Cancer Risk: Pooled Analysis of Couples from the IARC Multicentric Case-Control Studies. *Cancer Epidemiology, Biomarkers and Prevention*. Vol.20, No.7, pp.1379-1390, Epub 2011 May 24.

Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., et al. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature*, Vol.375, pp.503–506

McLaughlin-Drubin, M. & Münger, K. (2009a). The Human Papillomavirus E7 Oncoprotein. *Virology,* Vol.384, No.2, pp.335–344

McLaughlin-Drubin, M. & Münger, K. (2009b). Oncogenic Activities of Human Papillomaviruses. *Virus Research,* Vol.143, No.2, pp. 195–208

Mimica, M., Tomić, S., Kardum, G., Hofman, I.D., Kaliterna, V. & Pejković, L. (2010). Ki-67 quantitative evaluation as a marker of cervical intraepithelial neoplasia and human papillomavirus infection. *International Journal of Gynecological Cancer,* Vol.20, No.1, pp.116-119

Montag, M., Blankenstein, T., Shabani, N., Bru¨ning, A. & Mylonas, I. (2011) Evaluation of two commercialised in situ hybridisation assays for detecting HPV-DNA in formalin-fixed, paraffin-embedded Tissue. *Archives of Gynecology and Obstetrics,* Vol.284, pp.999–1005

Moscicki, A., Schiffman, M., Kjaer, S. & Villa, L. (2006) Chapter 5: Updating the natural history of HPV and anogenital cancer. *Vaccine*, Vol.24, No.S3, pp.42-51

Mulvany, N.J., Allen, D.G.& Wilson, S.M. (2008). Diagnostic utility of p16INK4a: a reappraisal of its use in cervical biopsies. *Pathology,* Vol.40, No.4, pp.335-344

Münger, K., Yee, C.L., Phelps, W.C., Pietenpol, J.A., Moses, H.L. & Howley, P.M. (1991). Biochemical and biological differences between E7 oncoproteins of the high- and low risk human papillomavirus types are determined by amino-terminal sequences. *Journal of Virology,* Vol.65, pp.3943–3948

Munoz, N., Bosch, F.X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K.V., et al. (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. *New England Journal of Medicine*, Vol.348, pp.518–527

Munoz, N., Castellsague, X., Berrington de Gonzalez, A. & Gissmann, L. (2006) Chapter 1: HPV in the etiology of human cancer. *Vaccine*, Vol.24, No.S3, pp.1-10

Murphy, N., Ring, M., Hefron, C.C., King, B., Killalea, A., Hughes, C., et al. (2005). p16INK4A, CDC6, and MCM5: predictive biomarkers in cervical preinvasive neoplasia and cervical cancer. *Journal of Clinical Pathology,* Vol.58, pp.525–534

Nakao, Y., Yang, X., Yokoyama, M., Ferenczy, A., Tang, S.C., Pater, M.M., et al. (1997). Induction of p16 during immortalization by HPV 16 and 18 and not during malignant transformation. *British Journal of Cancer*, Vol.75, No.10, pp.1410-1416

Nakashima, R., Fujita, M., Enomoto, T., Haba, T., Yoshino, K., Wada, H., et al. (1999). Alteration of p16 and p15 genes in human uterine tumours. *British Journal of Cancer,* Vol.80, pp.458-467

Negri, G., Vittadello, F., Romano, F., Kasal, A., Rivasi, F., Girlando, S., et al. (2004). P16INK4a expression and progression risk of low-grade intraepithelial neoplasia of the cervix uteri. *Virchows Archives*, Vol.445, pp.616–20

Negri, G., Bellisano, G., Zannoni, G.F., Rivasi, F., Kasal, A., Vittadello, F., et al. (2008). P16ink4a and HPV L1 immunohistochemistry is helpful for estimating the behavior of low-grade dysplastic lesions of the cervix uterii. *American Journal of Surgical Pathology,* Vol.32, No.11, pp.1715-1720

O'Neill, C. & McCluggage, G. (2006). p16 expression in the female genital tract and its value in diagnosis. *Advances in Anatomic Pathology*, Vol.13, pp.8–15

Ohtani, N., Yamakoshi, K., Takahashi, A. & Hara, E. (2004). The $p16^{INK4A}$-RB pathway: molecular link between cellular senescence and tumor suppression. *Journal of Medical Investigation*, Vol.51, pp.146-153

Ordi, J., Garcia, S., del Pino, M., Landol, S., Alonso, I., Quinto,' L., & Torne,' A. (2008). p16INK4a immunostaining identifies occult CIN lesions in HPV-positive women. *International Journal of Gynecological Pathology,* Vol.28, pp.90–97

Pei, X.F. (1996). The human papillomavirus E6/E7 genes induce discordant changes in the expression of cell growth regulatory proteins. *Carcinogenesis,* Vol.17, pp.1395–1401

Pett, M. & Coleman, N. (2007). Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? *Journal of Pathology*, Vol.212:, pp.356–367

Pinto, A., Schlecht, N., Woo, T., Crum, C.P. & Cibas, E. (2008). Biomarker (ProEx C, p16INK4A, and MiB-1) distinction of high-grade squamous intraepithelial lesion from its mimics. *Modern Pathology*, Vol.21, pp.1067–1074

Poljak, M. & Kocjan, B. (2010) Commercially available assays for multiplex detection of alpha human papillomaviruses Expert Rev. *Anti Infect Therapy,* Vol.8, No.10, pp.1139–1162

Prasad, C., Genest, D. & Crum, C.P. (1994). Nondiagnostic squamous atypia of the cervix (atypical squamous epithelium of undetermined significance): histologic and molecular correlates. *International Journal of Gynecological Pathology,* Vol.13, pp.220–227

Quelle, D., Zindy, F., Ashmund, R. & Sherr, C.J. (1995). Alternative reading frames of the INK4α tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell*, Vol.83, pp.993-1000

Ruas, M. & Peters, G. (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochimica et Biophysica Acta*, Vol.1378, pp.115-177

Salvia, P., Bergo, S., Bonesso-Sabadini, P., Tagliarini, E., Hackel, C. & De Angelo Andrade, L. (2004). Correlation between histological criteria and human papillomavirus presence based on PCR assay in cervical biopsies. *International Journal of Gynecological Cancer,* Vol.14, pp.126–132

Sanati, S., Huettner, P. & Ylagan, L.R. (2010). Role of ProExC: a novel immunoperoxidase marker in the evaluation of dysplastic squamous and glandular lesions in cervical specimens. *International Journal of Gynecological Pathology,* Vol.29, No.1, pp.79-87

Sano, T., Oyama, T., Kashiwabara, K., Fukuda, T. & Nakajima, T. (1998). Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions *American Journal of Pathology,* Vol.153, pp.1741–1748

Sarmadi, S., Izadi-Mood, N., Pourlashkari, M., Yarandi, F. & Sanii, S. (2011). HPV L1 capsid protein expression in squamous intraepithelial lesions of cervix uteri and its relevance to disease outcome. *Archives of Gynecology and Obstetrics,* Jul 26. [Epub ahead of print]

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J. & Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*, Vol.63, pp.1129–1136

Scheurer, M.E., Guillaud, M., Tortolero-Luna, G., McAulay, C., Follen, M. & Adler-Storthz, K.(2007). Human papillomavirus-related cellular changes measured by cytometric analysis of DNA ploidy and chromatin texture. *Cytometry B Clinical Cytometry,* Vol.72, pp.324–331

Schmidt, D., Bergeron, C., Denton, K.J., Ridder, R.; European CINtec Cytology Study Group. (2011). p16/ki-67 dual-stain cytology in the triage of ASCUS and LSIL papanicolaou cytology: results from the European equivocal or mildly abnormal Papanicolaou cytology study. *Cancer Cytopathology,* Vol.119, No.3, pp.158-166

Serrano, M. (1997). The Tumor Suppressor Protein $p16^{INK4a}$. *Experimental Cell Research*, Vol.237, pp.7–13Shi, J., Liu, H., Wilkerson, M., Huang, Y., Meschter, S., Dupree, W., Schuerch, C. & Lin, F. (2007). Evaluation of p16INK4a, minichromosome maintenance protein 2, DNA topoisomerase IIalpha, ProEX C, and p16INK4a/ProEX C in cervical squamous intraepithelial lesions. *Human Pathology,* Vol.38, pp.1335-1344

Snijders, P., Steenbergen, R., Heideman, D., & Meijer, C. (2006). HPV-mediated cervical carcinogenesis: concepts and clinical implications. *Journal of Pathology,* Vol.208, pp.152–164

Snijders, P.J., Heideman, D.A. & Meijer, C.J. (2010). Methods for HPV detection in exfoliated cell and tissue specimens. *APMIS,* Vol.118, No.6-7, pp.520-528

Southern, S., McDicken, I. & Herrington, C.S. (2000). Evidence for keratinocyte immortalization in high-grade squamous intraepithelial lesions of the cervix infected with high-risk human papillomaviruses. *Laboratory Investigation,* Vol.80, pp.539–544

Stanley, M. (2010). HPV - immune response to infection and Vaccination. *Infectious Agents and Cancer,* Vol.5, pp.19

Straight, S.W., Hinkle, P.M., Jewers, R.J. & McCance, D.J. (1993). The E5 Oncoprotein of Human Papillomavirus Type 16 Transforms Fibroblasts and Effects the Downregulation of the Epidermal Growth Factor Receptor in Keratinocytes. *Journal of Virology,* Vol.67, No.8, pp.4521–4532

Talbert-Slagle, K. & DiMaio, D. (2009). The bovine papillomavirus E5 protein and the PDGF beta receptor: it takes two to tango. *Virology*, Vol.384, No.2, pp.345–351

Tambouret, R.H., Misdraji, J. & Wilbur, D.C. (2008). Longitudinal clinical evaluation of a novel antibody cocktail for detection of high-grade squamous intraepithelial lesions on cervical cytology specimens. *Archives of Pathology and Laboratory Medicine,* Vol.132, No.6, pp.918-925

Tan, G.C., Norlatiffah, S., Sharifah, N.A., Razmin, G., Shiran, M.S., Hatta, A.Z. & Paul-Ng, H.O. (2010). Immunohistochemical study of p16 ^{INK4A} and survivin expressions in cervical squamous neoplasm. *Indian Journal of Pathology and Microbiology,* Vol.53, pp.1-6

The FUTURE I/II Study Group. (2010). Four year efficacy of prophylactic human papillomavirus quadrivalent vaccine against low grade cervical, vulvar, and vaginal intraepithelial neoplasia and anogenital warts: randomised controlled trial. *BMJ,* Vol.340, pp.c3493

Tsoumpou, I., Arbyn, M., Kyrgiou, M., Wentzensen, N., Koliopoulos, G., Martin-Hirsch, P., Malamou-Mitsi, V. & Paraskevaidis, E. (2009). p16INK4a immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis. *Cancer Treatment Reviews,* Vol.35, No.3, pp.210–220

Van Niekerk, D., Guillaud, M., Matisic, J., Benedet, J., Freeberg, J., Follen, M., et al. (2007). p16 and MIB1 improve the sensitivity and specificity of the diagnosis of high grade squamous intraepithelial lesions: Methodological issues in a report of 447 biopsies with consensus diagnosis and HPV HCII testing. *Gynecologic Oncology,* Vol.107, pp.S233–240.

Volgareva, G., Zavalishina, L., Andreeva, Y., Frank, G., Krutikova, E., Golovina, D. et al. (2004). Protein p16 as a marker of dysplastic and neoplastic alterations in cervical epithelial cells. *BMC Cancer,* Vol. 4, pp.58

Voss, J.S., Kipp, B.R., Campion, M.B., Sokolova, I.A., Henry, M.R., Halling, K.C. & Clayton, A.C.. (2009). Comparison of fluorescence in situ hybridization, hybrid capture 2 and polymerase chain reaction for the detection of high-risk human papillomavirus in cervical cytology specimens. *Analytical and Quantitative Cytology and Histology*, Vol.31, No.4, pp.208-216

Walboomers, J., Jacobs, M., Manos, M., Bosch, F.X., Kummer, J.A., Shah, K.V., Snijders, P.J.F., Peto, J., Meijer, C. & Munoz, N. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology*, Vol.189, pp.12–19

Walts, A.E. & Bose, S. (2009). p16, Ki-67, and BD ProExC immunostaining: a practical approach for diagnosis of cervical intraepithelial neoplasia. *Human Pathology,* Vol.40, No.7, pp.957-964,Epub 2009 Mar 9

Wang, S.S., Trunk, M., Schiffman, M., Herrero, R., Sherman, M., Burk, R., et al. (2004). Validation of p16INK4a as a marker of oncogenic human papillomavirus infection in cervical biopsies from a population-based cohort in Costa Rica. *Cancer Epidemiology, Biomarkers and Prevention,* Vol. 13, pp.1355–1360

Whiteside, M., Siegel, E. & Unger, E. (2008). Human Papillomavirus and Molecular Considerations for Cancer Risk. *Cancer,* Vol.113, No.10 suppl, pp.2981–2994

Wright, T.C. (2006). Pathology of HPV infection at the cytologic and histologic levels: basis for a 2-tiered morphologic classificationsystem. *International Journal of Gynecology and Obstetrics*, Vol.94, No.suppl 1, pp.22–31

Yoshida, T., Sano, T., Kanuma, T., Owada, N., Sakurai, S., Fukuda, T. & Nakajima, T. (2008). Immunochemical analysis of HPV L1 capsid protein and p16 protein in liquid-based cytology samples from uterine cervical lesions. *Cancer*, Vol.114, No.2, pp.83-88

Yu, L., Wang, L., Zhong, J. & Chen, S. (2010). Diagnostic Value of p16INK4A, Ki-67, and Human Papillomavirus L1 Capsid Protein Immunochemical Staining on Cell Blocks From Residual Liquid-Based Gynecologic Cytology Specimens. *Cancer (Cancer Cytopathology),* Vol.118, pp.47–55

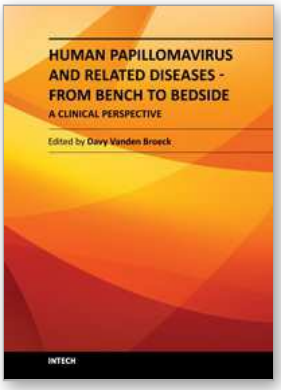
Zerfass, K., Schulze, A., Spitkovsky, D., Friedman, V., Henglein, B. & Jansendurr P. (1995). Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. *Journal of General Virology*, Vol.69, pp.6389–6399

Zheng, L., Liu, A.L., Qi, T., Wang, Q., Cai, Z., Su, Y.J., Hu, Y.W., Liu, G.B. & Wei, L.H. (2010). Human telomerase RNA gene amplification detection increases the specificity of cervical intraepithelial neoplasia screening. *International Journal of Gynecological Cancer,* Vol.20, No.6, pp.912-917

zur Hausen, H. (1977). Human papilloma viruses and their possible role in squamous cell carcinomas. *Current Topics in Microbiology and Immunology*, Vol.78, pp.1–30

zur Hausen, H. (2008). Papillomaviruses—to Vaccination and Beyond. *Biochemistry (Moscow),* Vol.73, No.5, pp.498-503

zur Hausen, H. (2009). Papillomaviruses in the causation of human cancers — a brief historical account. *Virology,* Vol.384, pp.260–265



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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on the clinical aspects of HPV and related disease, highlighting the latest developments in this field.

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