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Mini Review

The clinical impact of using p16^{INK4a} immunochemistry in cervical histopathology and cytology: An update of recent developments

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Cervical cancer screening test performance has been hampered by either lack of sensitivity of Pap cytology or lack of specificity of Human Papillomavirus (HPV) testing. This uncertainty can lead to unnecessary referral and treatment, which is disturbing for patients and increases costs for health care providers. The identification of p16^{INK4a} as a marker for neoplastic transformation of cervical squamous epithelial cells by HPVs allows the identification of HPV-transformed cells in histopathology or cytopathology specimens. Diagnostic studies have demonstrated that the use of p16^{INK4a} immunohistochemistry substantially improves the reproducibility and diagnostic accuracy of histopathologic diagnoses. p16^{INK4a} cytology has substantially higher sensitivity for detection of cervical precancer in comparison to conventional Pap tests. Compared to HPV DNA tests, immunochemical detection of p16^{INK4a}-stained cells demonstrates a significantly improved specificity with remarkably good sensitivity. About 15 years after the initial observation that p16^{INK4a} is overexpressed in HPV-transformed cells we review the accumulated clinical evidence suggesting that p16^{INK4a} can serve as a useful biomarker in the routine diagnostic work up of patients with HPV infections and associated lesions of the female anogenital tract.

Cervical cancer screening tests aim to identify women with cervical precancerous lesions referred to as High Grade Squamous Intraepithelial Lesions (HSIL), who are at increased risk to develop invasive carcinomas. Women with abnormal screening test results are referred to colposcopy usually after some type of triage test. If HSIL is confirmed by biopsies taken during colposcopy, the lesion is removed to prevent progression to invasive cervical cancer. Population wide screening with the Pap test has been used in many Western countries and has led to a substantial reduction of the

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Magnus von Knebel Doeberitz was co-founder, shareholder, and member of the board of mtm-laboratories AG, a privately held company that developed and purchased products related to the content of this manuscript. mtm was acquired by Roche in 2011.

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incidence and mortality of cervical cancer (reviewed in http://www.iarc.fr/en/publications/pdfs-online/prev/handbook10/handbook10-chap2.pdf and citations therein). However, the Pap test is prone to misinterpretation[1] and its sensitivity is limited.[2,3] Countries that established cervical cancer screening programs recommend frequent repeated tests to compensate for the established limitations in diagnostic sensitivity and to safeguard the protective effects of the cervical cancer screening program.

In the early 1980s, human papillomavirus (HPV) infections were identified as the predominant risk factor for cervical cancer.[4] Since then, many sensitive tests have been developed to detect HPV infections in the female genital tract.[3,5] However, since HPV infections are very widespread especially in younger women, HPV tests do not have a high positive predictive value for the presence of HSILs. Women who test positive for HPV therefore require a further triage test as for example a Pap cytology test or more recently developed biomarker-based triage tests.[6–9]

On the basis of a refined understanding of the molecular pathogenesis how HPVs contribute to the neoplastic transformation of cervical squamous epithelial cells, biomarker-based test systems have been developed and clinically evaluated. In particular, the identification of p16INK4a as marker for "transforming" HPV infections promises to add more accuracy in cancer early detection and diagnostic programs. The current state of the clinical development of this biomarker in cervical cancer screening will be reviewed in this summary.

Pathogenesis of HPV-triggered neoplastic lesions: The detection of p16INK4a as biomarker for transforming HPV infections

HR-HPV infections and in particular infections by HPV types 16 and 18 have been identified as the primary risk factor for cervical cancer (reviewed in Ref. 4). These infections are very widespread among young men and women and usually resolve spontaneously.[10] Only few of the initially infected individuals ever develop neoplastic lesions.[11,12] The vast majority of neoplastic lesions caused by HPV-infections are located in the cervical squamo-glandular junction.[13] This implies that distinct cells located within this zone are particularly prone for the development of HPV-related cancers.[14] Recent evidence suggests that these cells may be derived from remaining embryonic epithelial cells.[15]

The role of HPV in cervical carcinogenesis has been well defined.[16] The key step in the pathogenesis of HPV-linked cancers is the activation of the viral oncogenes E6 and E7 in the basal and para-basal cells of the infected epithelium (reviewed in Ref. 14 and citations therein). These viral genes if expressed in basal or parabasal cells trigger chromosomal instability and major numerical and structural alterations of the host cell chromosomes.[17,18] This leads to uneven distribution of the overall DNA content, aneuploidy and is reflected by shifts of the nuclear staining pattern, the staining intensity and finally the overall morphology of the nuclei of transformed squamous epithelial cells. These morphological changes triggered by an HPV infection formed the basis of the Pap-test (reviewed in Ref. 19).

About 1 in 5 women younger than 30 years of age has an HR-HPV infection; however, most of them regress spontaneously, probably due to natural immune responses that apparently develop during the normal course of these infections.[20–22] The rate of infections in women older than 30 years of age substantially decreases and ranges somewhere around 5–10%.[11–13]

HPV infections are usually classified as acute, self-limited or persistent. Whereas about 90% of acute infections usually resolve spontaneously within several months, about 10% persist.[11] The term "persistent infection" is only loosely defined. It is generally used if in any individual, the same HPV type has been detected on two or more occasions, usually with an interval of at least 6 months.[23] This classification neglects the biological activities of the virus in its host cells. All HPV-induced pathologies depend on the different patterns of HPV gene expression in their target cells. It may thus be reasonable to use a terminology that better addresses the respective molecular gene expression profiles of oncogenic human papillomaviruses in the pathogenesis of cervical lesions.

Conceptually at least two different phases in the evolution of HPV infections may be distinguished that are characterized by specific viral gene expression patterns,[14,24,25] (Fig. 1):

The transient, permissive or productive phase is characterized by well controlled, very low level expression of the E6 and E7 genes in basal and para-basal cells of the squamous epithelium. If these basal cells start differentiating and progress during the normal differentiation pathway upward to the intermediate cell layer, the squamous cells lose their capacity to proliferate and irreversibly exit the cell cycle. In these maturated senescent squamous epithelial cells, the papillomavirus genes become expressed at higher rates and trigger the replication of episomal viral genomes within the nuclei of the infected cells. If these cells reach the superficial cell layer the virus shifts its expression pattern to the late genes E4, L1 and L2 whose gene products have not been found in the lower layers of the squamous epithelium.[26–28] The late gene products permit packaging of the replicated viral genomes and the newly produced viral particles are released from disintegrating keratinocytes at the surface of the infected squamous epithelium.

The transforming phase of HPV infections is characterized by marked overexpression of the E6 and E7 genes in the basal and parabasal squamous cells that have escaped the regulatory control by the E2 protein.[14,24,25] This triggers chromosomal instability and may allow for the selection of preneoplastic cell clones that may eventually progress into invasive carcinomas. Cells displaying the transforming mode of viral gene expression may initially arise among a majority of cells displaying the productive mode of viral gene

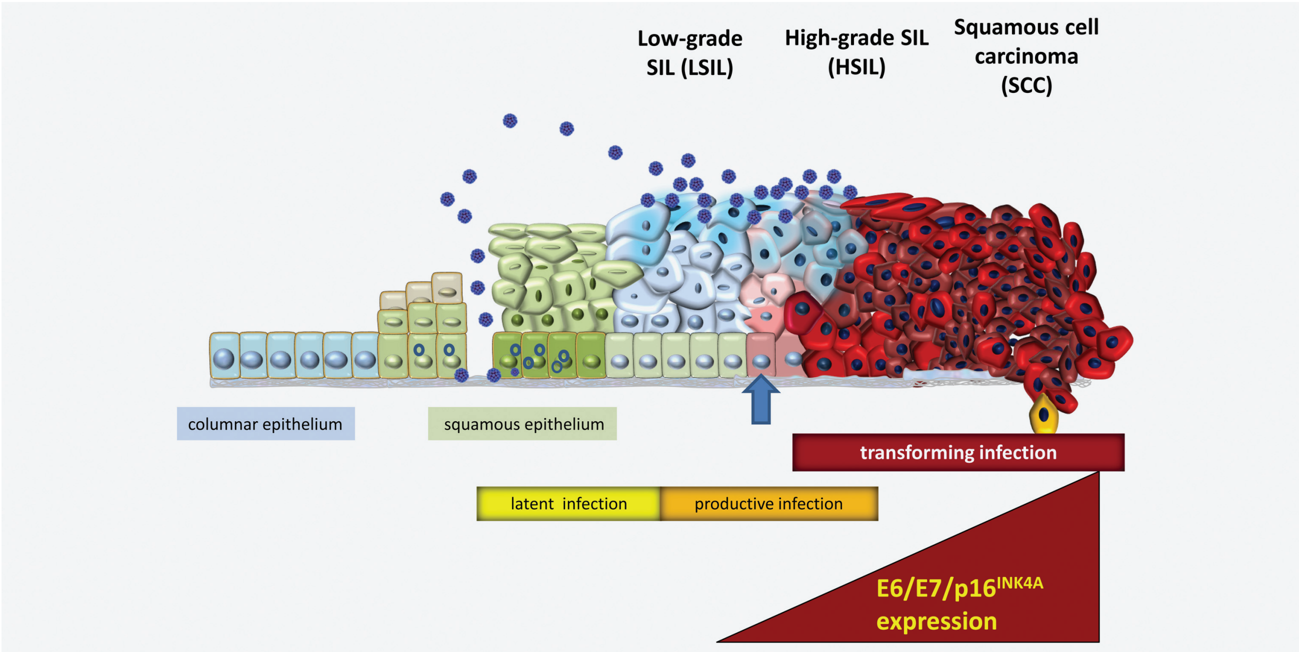

Figure 1. Schematic representation of HPV infection stages in the cervical epithelium. *Via* minor lacerations the virus enters its host cell in the basal or para-basal epithelium. Differentiation of infected host cells goes along with replication of the viral genome and subsequent release of new viral particles from the superficial differentiated cells (productive infection), which is cytologically/histologically characterized as low grade squamous intraepithelial lesion (LSIL). Incident overexpression of the viral oncogenes E6/E7 (transforming infection) disrupts cell cycle control and may lead to high grade squamous intraepithelial lesion (HSIL) or subsequent squamous cell carcinoma (SCC). Transforming HPV infections are characterized by overexpression of p16^{INK4a}. Modified from von Knebel Doeberitz and Vinokurova, 2009.

expression. Therefore, it is important to note that cells in the early transforming phase still retain the capacity to undergo squamous epithelial differentiation and, thus, also viral replication.[29] Respective lesions therefore appear as LSIL or CIN1 lesions. Only if these cells expand and overgrow the cells displaying the productive mode of viral gene expression, respective lesions appear more advanced as HSIL or CIN 2+.

Recent work has demonstrated that high level expression of E7 triggers oncogenic stress signals and induces epigenetic remodeling particularly of the CDKN2A (p16^{INK4a}/ARF) locus that results in substantial overexpression of the p16^{INK4a} protein.[30] The p16^{INK4a} is a cyclin dependent kinase inhibitor that blocks the phosphorylation of various cyclins and counteracts the phosphorylation and inactivation of pRB[31]. Its overexpression usually occurs in cells of aged organisms and p16^{INK4a} is increasingly expressed in aging tissues.[32,33] In normal somatic cells overexpression of p16^{INK4a} results in immediate cell cycle arrest and irreversible chromatin condensation (reviewed in Ref. 34 and citations therein). Thus, p16^{INK4a} protects cells incurring genomic damages from further proliferation and expansion. Growth inhibitory functions of p16^{INK4a} are predominantly mediated by its cyclin dependent kinase activity inhibiting the cyclin dependent kinase 4 (CDK 4) that essentially prevents hyperphosphorylation and, thus, inactivation of the pRB protein.[31] In many human neoplasms including breast, pancreatic, colon cancers as well as malignant melanomas, the p16^{INK4a} gene function is lost by gene deletions, mutations or epigenetic silencing.[35] In other tumors, its growth arresting function may be abolished by inactivating downstream inhibitory signals for example by inactivating pRB functions. As pRB is also inhibited by the HPV E7 protein, all cells transformed by oncogenic papillomaviruses are no longer able to control their cell cycle *via* the pRB pathway (Fig. 2). These cells usually proliferate and substantially overexpress p16^{INK4a}, which can be detected by immunohistochemistry as a strong diffuse overexpression of p16^{INK4a} that is now recognized to be the hallmark of HPV-induced transformation (Figs. 3*a* and 3*b*,B) (for review see Ref. 36 and references cited therein). Furthermore, the simultaneous detection of a proliferation marker like Ki-67 together with p16^{INK4a} in the same cell is a specific sign of neoplastic transformation (Fig. 3*b*).[37] Staining for both, p16^{INK4a} and Ki-67 can thus be used to identify single HPV-transformed cells in cytology specimens (reviewed in Ref. 36 and citations therein) (Fig. 3*c*). Interestingly, recent evidence further suggests that high level expression of p16^{INK4a} is also required to maintain the neoplastic growth of HPV-transformed cells.[38]

The clinical impact of using p16^{INK4a} immunohistochemistry in histology

Historically, cervical precancerous lesions have been classified as Cervical Intraepithelial Neoplasia (CIN) grade 1–3.[39,40] This definition was based on the assumption that all CIN lesions

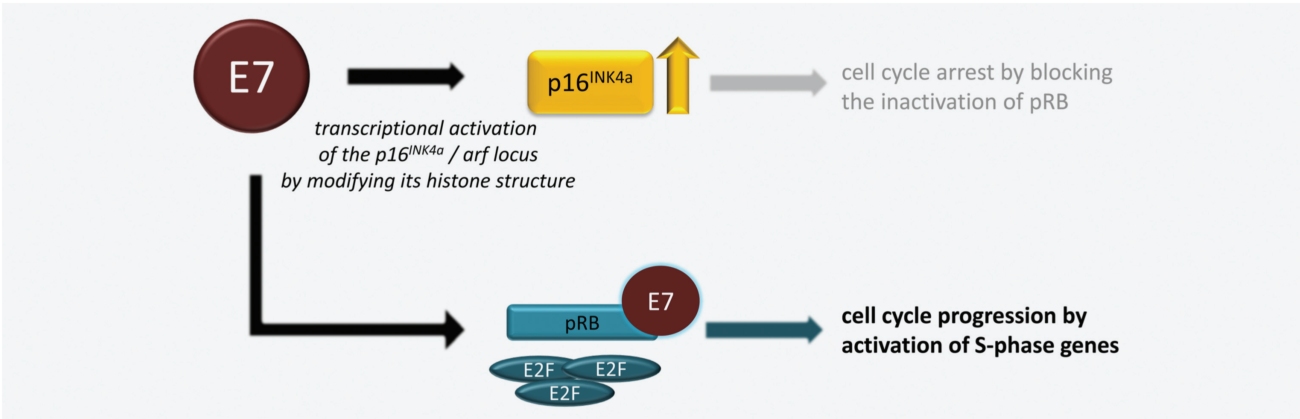

Figure 2. Scheme of HPV E7 effects on $p16^{INK4a}$ expression levels and cell cycle regulation (black arrows). E7 mediates $p16^{INK4a}$ overexpression *via* transcriptional activation. High $p16^{INK4a}$ levels would normally result in cell cycle arrest (grey arrow and text in figure). However, at the same time E7 disrupts pRB and thereby cell cycle progression is triggered (lower part of the figure) despite high $p16^{INK4a}$ levels.

progress gradually from CIN 1 to CIN2 and 3. A recent consensus conference that aimed to unify the terminology of HPV-associated squamous lesions of the lower anogenital tract was strongly influenced by the biological aspects of the various stages of HPV-infections and their relation to biomarker expression (Lower Anogenital Squamous Terminology (LAST) Project).[41] A two tiered classification system was therefore proposed, in part paralleling the Bethesda classification for cervical cytologic abnormality, differentiating low grade squamous epithelial lesions (LSIL) from high grade squamous intraepithelial lesions (HSIL). LSIL represents the productive phase of an oncogenic HPV infection and HSIL represents the more advanced transforming phase of the infections in that diffuse $p16^{INK4a}$ stained abnormal cells expand beyond the lower third of the thickness of the affected epithelium (Figs. 1 and 3*a*). Use of $p16^{INK4a}$ was recommended to adjudicate inconclusive histology results[40–44] (Fig. 4).

A positive $p16^{INK4a}$ histology stain is defined as strong and diffuse staining of the basal and para-basal squamous cell compartment at least for the lower third of the epithelial thickness in well oriented sections (Fig. 3*a*).[45] Full thickness staining or extension into the upper third or upper half is often found in HSIL. The application of $p16^{INK4a}$ immunohistochemistry (IHC) with these criteria substantially improves the inter-observer reproducibility and accuracy of histopathology diagnoses of cervical lesions.[45–49] $p16^{INK4a}$-negative CIN2+ lesions are likely morphological mimics of high grade lesions such as immature squamous metaplasia or early dysplastic alterations(LSILs) that have not entered the transforming HPV infection stage. Consequently, in a study that used $p16^{INK4a}$ immunohistochemistry to adjudicate the H&E based diagnoses of cervical biopsies, Pap test results and HPV-testing showed improved sensitivity and specificity.[50]

Figure 4 outlines the current recommendations that are discussed in greater detail in Refs. 41 and 43. In summary, LAST does not recommend using the $p16^{INK4a}$ IHC in case of clearly normal or LSIL/CIN1 histology and unequivocal CIN3. LAST recommends using the $p16^{INK4a}$ IHC for the differential diagnosis between HSIL and histopathologic micmics of precancers, such as immature metaplasia, atrophy or reparative epithelial changes (Figs. 4 and 5). Further, LAST recommends using $p16^{INK4a}$ IHC if the pathologist is entertaining an H&E morphologic interpretation of CIN 2 (under the old terminology) to decide whether the lesion should be called LSIL ($p16^{INK4a}$-negative) or HSIL (p16-positive in more than one third of the epithelium) (Fig. 4). Finally, LAST recommends using the $p16^{INK4a}$ IHC as an adjudication tool for cases in which there is professional disagreement in interpretation, with the caveat that the differential diagnosis includes a precancerous lesion.[49]

At the moment, $p16^{INK4a}$ immunohistochemistry is not recommended in case of unequivocal CIN 1 or LSIL. Importantly, half of CIN1 show diffuse and strong $p16^{INK4a}$ staining.[51] According to LAST, these lesions should still be interpreted as LSIL, despite the diffuse $p16^{INK4a}$ staining. Some studies have suggested that CIN 1 that do not progress are mostly $p16^{INK4a}$ negative, whereas CIN 1 that progress to HSIL are more likely $p16^{INK4a}$ positive.[52–57]. These reports support the notion that the activation of the viral oncogenes E6 and E7 in basal squamous epithelial cells as evidenced by $p16^{INK4a}$ overexpression initiates the transformation cascade but does not preclude that the affected lesions may still regress. However, they further underline the notion that on the molecular level the shift from a productive infection to a transforming infection occurs in single cells within the LSIL or CIN1 lesions. These cells are highlighted by the enhanced expression of $p16^{INK4a}$ and apparently gain a selective advantage and may subsequently overgrow their neighboring cells. The fact that many of these lesions still appear to regress spontaneously clearly

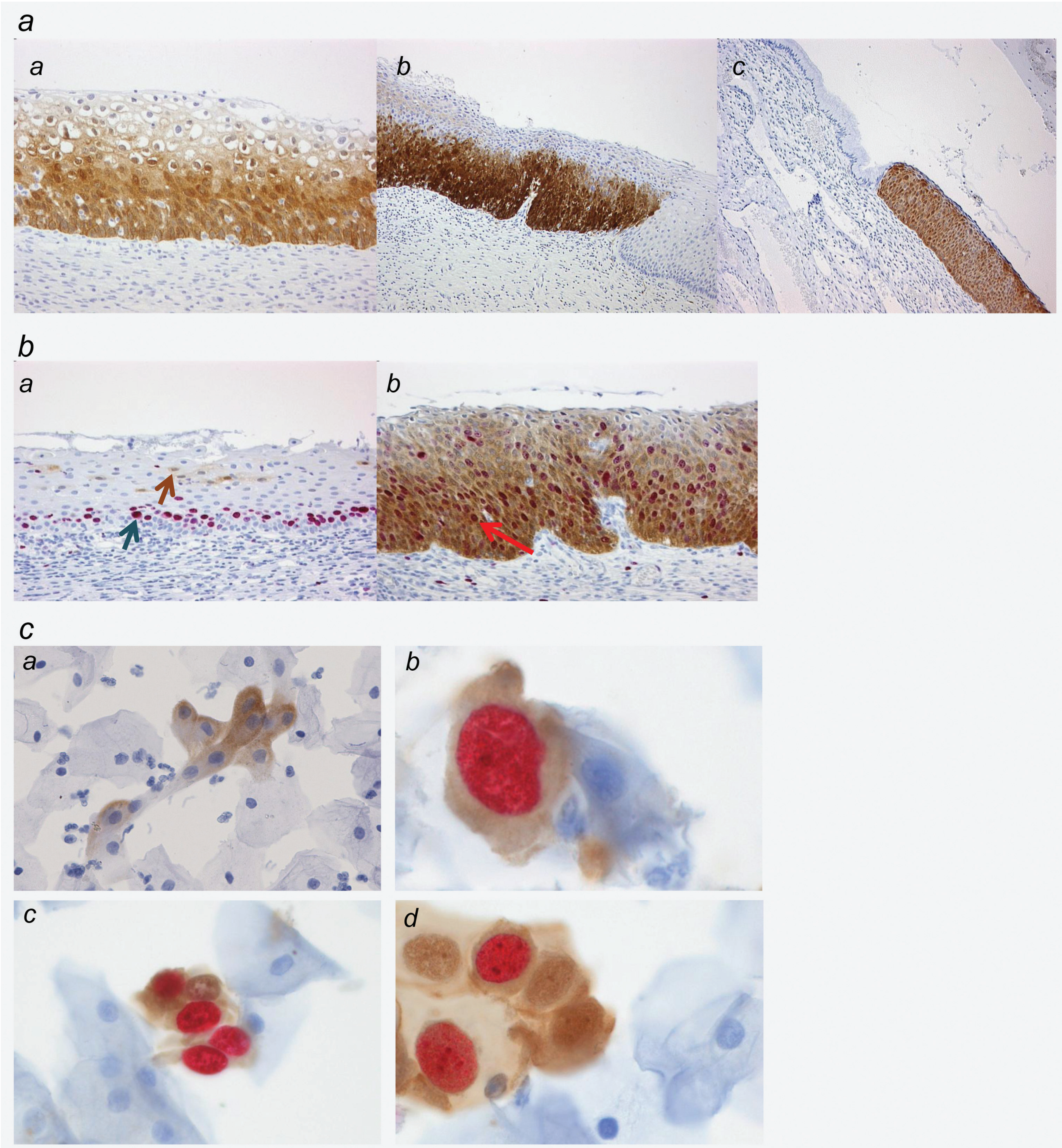

Figure 3. (*a*) p16^{INK4a} immunohistochemical staining of cervical biopsies. Examples of diffuse positive stains indicating transforming HPV infections in (*a*) LSIL (CIN1), (*b*) HSIL (CIN2) and (*c*) (CIN3). (*b*) p16^{INK4a}/Ki-67 dual stain of cervical biopsies. (*a*) Dual-stain-negative normal epithelium with single p16^{INK4a}-expressing cells (brown arrow) and parabasal Ki-67 expression (blue arrow), however no cells co-expressing both markers arguing against transforming HPV infection. (*b*) Diffuse p16^{INK4a} expression (brown) in a dual stain-positive (red nuclei in brown cells) CIN3 indicating transforming HPV infection. (*c*) CINtec® PLUS Dual staining cytology visualizing p16^{INK4a} and Ki-67 in cervical cytology slides. A: p16^{INK4a}-expressing metaplastic cells (brown) without Ki-67 expression, indicating no proliferation and thus sustained cell cycle control arguing againt transforming HPV infection. b, c, d: p16^{INK4a}/Ki-67 dual-stain-positive (read nuclei, bown cytoplasm) cells, indicating transforming HPV infections.

indicates that not all initially transformed cells inevitably will progress to HSIL or even cancer. In contrast, it is likely that a majority of them still retain the capacitiy to regress, presumably due to immunological interference. At the moment, the data are not sufficient to warrant different management of p16-positive versus p16-negative CIN1.

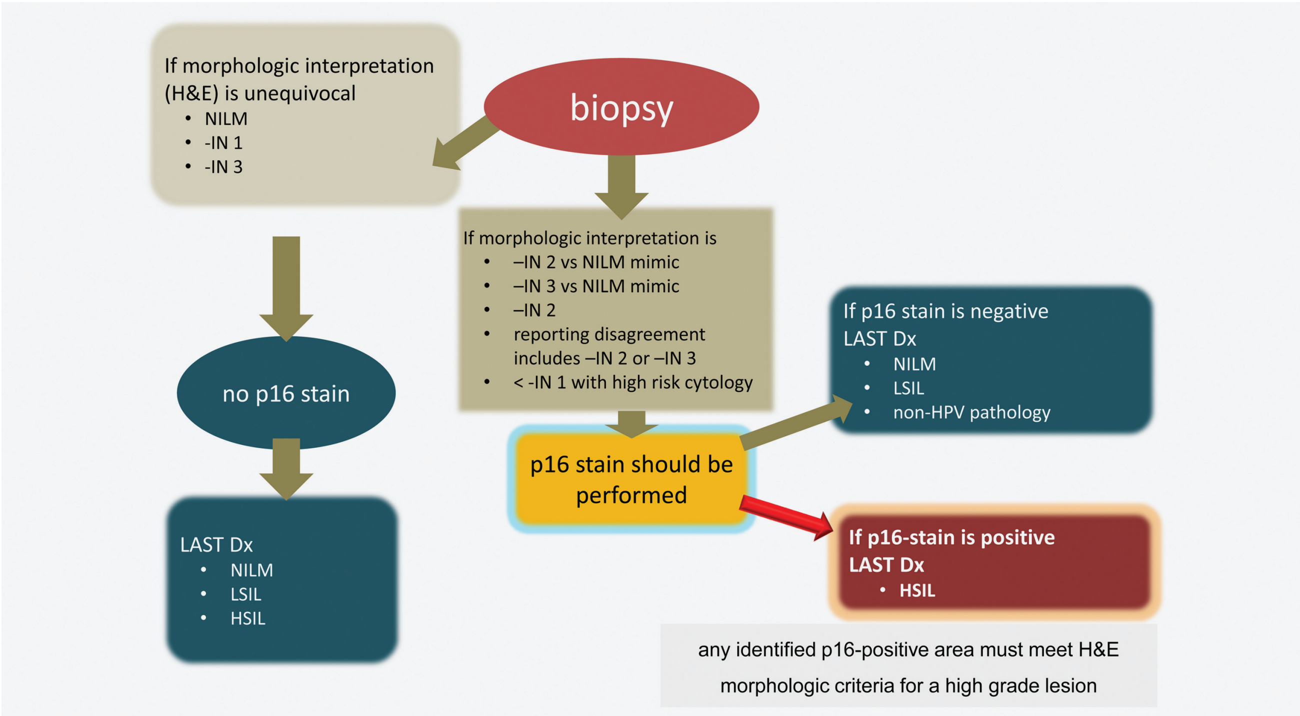

Figure 4. Decision flow-chart of adding p16INK4a immunohistochemistry to cervical biopsy interpretation according to LAST Project recommendations (a joint project of the college of American pathologists (CAP) and the American Society of Colposcopy and Cervical Pathology (ASCCP).[40]

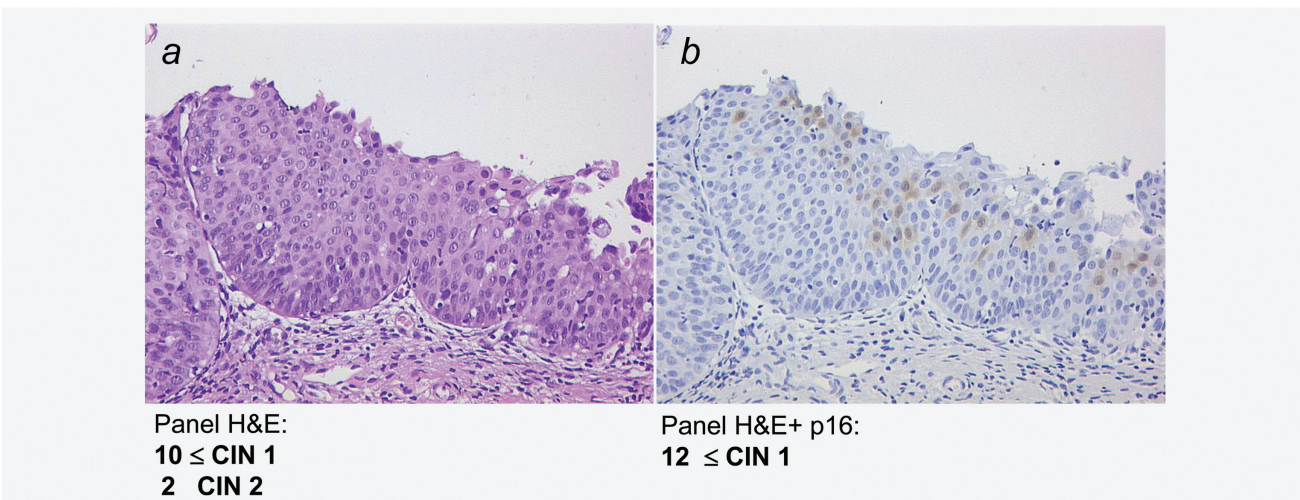

Figure 5. H&E stain (*a*) and p16INK4a immunohistochemistry (*b*) of a cervical biopsy and pathologists diagnoses. While 2 pathologists rendered the diagnosis of CIN2 based on the H&E stain, complete consensus for ≤CIN1 was reached among 12 pathologists when interpreting p16INK4a immunohistochemistry in conjunction with H&E. p16INK4a immunohistochmcal staining is negative for a diffuse pattern (*b*).

Large prospective randomized trials with follow-up of p16INK4a-positive and negative LSIL (CIN 1) to define the progression risk of each group need to be performed before giving recommendations to the pathologists and clinicians in this particular setting.

The clinical impact of using p16INK4a in triaging minor cytological atypia

p16INK4a immunocytochemistry. The availability of a marker that provides a similar sensitivity as HPV testing, but with a significantly higher specificity would be highly desirable to improve current triage strategies for equivocal cytology results like atypical squamous cells of undetermined significance (ASC-US) and low grade squamous intraepithelial lesions (LSIL), thereby reducing colposcopy referral rates. Various studies have been performed to evaluate p16^{INK4a} immunocytochemical staining also in comparison to HPV testing (Table 1). A retrospective analysis on a large cohort of cytology cases categorized as ASC-US or LSIL used adjudicated consensus histology of cervical biopsy tissues as reference standard and p16^{INK4a} cytology to identify HSIL (CIN2+).[58] The p16^{INK4a} cytology showed a sensitivity and specificity in ASC-US of 92.6% (84.6–97.2) and 63.2% (57.5–68.6) and in LSIL of 92.0% (86.1–95.9) and 37.1% (31.4–43.0), respectively. HPV testing performed in the same population showed a sensitivity and specificity for ASC-US of 90.1% (81.5–95.6) and 37.8% (32.4–43.5), and for LSIL of 95.7% (91.0–98.4) and 18.5% (14.2–23.5), respectively. In most studies, a similar sensitivity as HPV testing, but at a substantially higher specificity rate has been reported for p16^{INK4a} cytology when used for the triage of ASC-US, LSIL.[59] However, p16^{INK4a} single-staining immunocytochemistry protocols require morphologic interpretation of stained cells to distinguish between p16^{INK4a}-positive abnormal cells and those cervical cells occasionally over-expressing p16^{INK4a} due to physiological reasons, such as squamous atrophy or squamous metaplasia or endocervical or endometrial cells (Fig. 3*c*).[59,60]

Simultaneous detection of p16^{INK4a} and Ki-67 expression. The clinical performance of a novel approach, that is, the simultaneous detection of p16^{INK4a} and Ki-67 expression within the same cervical epithelial cell (referred to as p16^{INK4a}/Ki-67 dual stain cytology) as a morphology-independent marker of cell-cycle deregulation has been evaluated in the triage of ASC-US and LSIL cytology results (Fig. 3*c*) (Table 1).

In the same population as the one of Denton and coworkers.[58] using the residual material, the sensitivity of p16^{INK4a}/Ki-67 dual stained cytology for biopsy-confirmed CIN2+ was 92.2% (83.8–97.1) for ASC-US and 94.2% (88.8–97.4) for LSIL, whereas specificity rates were 80.6% (75.6–85.1) for ASC-US and 68.0% (62.2–73.4) for LSIL, respectively.[61] Similar sensitivity/specificity profiles were found for both women aged <30 years as well as women aged ≥ 30 years.[61] In this study, p16^{INK4a}/Ki-67 dual-stained cytology provides a similar sensitivity level as p16^{INK4a} alone and HPV testing for detecting underlying HSIL, whereas the specificity using this morphology-independent dual biomarker approach was higher compared with p16^{INK4a} cytology alone and significantly higher when compared with HPV testing.[61] In a study conducted on a population referred for colposcopy mainly because of abnormal cytology, sensitivity of dual staining was 86.4% (81.5–90.2) for CIN2+ and 93.2% (85.3–97.2) for CIN3+ while specificity was 59.5% (54.2–64.5) for CIN2+ and 60.1% (54.9–65.1) for CIN3+.[62] The p16^{INK4a}/Ki-67 sensitivity and specificity were lower than in the previous study but with still a significantly better specificity than HPV testing. Recently, these data were confirmed by Uijterwaal *et al.* who reported that p16/Ki-67 dual-stained cytology showed a sensitivity of 100%, a specificity of 64.4% and a negative predictive value (NPV) of 100% for CIN3.[63] Human papillomavirus testing of the same cohort demonstrated similar sensitivity (96.3%), and NPV (99.1%), but a significantly lower specificity (57.6%) for CIN3. Sensitivity, specificity and NPV for CIN2 of dual-stained cytology were 89.7, 73.1 and 95.1%, respectively, that was similar when compared with HPV testing. Importantly, during long-term follow-up, no CIN3 lesions developed in HPV positive, dual-stained negative women in this study.[63] The comparable sensitivity and NPV of dual-stained cytology for CIN3, combined with a significantly higher specificity, makes p16INK4a/Ki-67 dual-stained cytology indeed an interesting alternative to HPV testing for triaging ASC-US or LSIL cytology results.

p16^{INK4a} in the triage of HPV-positive women

Randomized controlled trials of HPV testing have repeatedly shown earlier detection of persistent HSIL compared with cytology[64–66]. However, directly referring to colposcopy alI HPV-positive women results in a marked increase in the number of colposcopies needed to detect a precancerous lesion.[3,67,68] Therefore, methods are needed for selecting, among HPV-positive women, those who have very low probability of carrying a colposcopy-detectable precancerous lesion and therefore not needing immediate colposcopy versus those that should be referred to colposcopy immediately.[7] p16^{INK4a} over-expression is a candidate biomarker for the triage of HPV-positive women (Table 2). It was first evaluated in a study nested in one of the 2 phases of the "New technologies for cervical cancer screening" (NTCC) randomized controlled trial, during which all women in the experimental arm were tested for high-risk HPV DNA by Hybrid Capture 2 and referred to colposcopy if positive.[68] Samples were taken at that moment and studied for p16^{INK4a} over-expression by immunostaining, which was not used for clinical decisions.[8] Among women aged 35–60 years at recruitment, the sensitivity of p16^{INK4a} overexpression was 92% (79–98) for CIN2+ and 86% (65–97) for CIN3+. This high sensitivity translates to a high NPV, providing high reassurance that HPV positive, p16^{INK4a}-negative women do not need immediate colposcopy. The relative sensitivity *versus* cytology when referring to colposcopy only HPV positive women who were also p16^{INK4a}-positive was 1.53 (1.15–2.02), almost the same obtained by referring to colposcopy all HPV positive women (1.63; 1.25–2.12). In contrast, among women aged 35–60 years, the specificity of p16^{INK4a} immunostaining among HPV-positive women was 57% (51–63) for CIN2+ and 56% (50–61) for CIN3+. If only the women who were both HPV and p16^{INK4a} positive were referred to colposcopy, then the referral rate would have been similar to that observed with cytology (ratio 1.08; 0.96–1.21), whereas with direct referral

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Table 1. Cross-sectional sensitivity and specificity of p16 or p16/ki67 *versus* HPV DNA in the triage of ASC-US and LSIL to identify CIN2+

Study	Test	Population	Sample size	Sensitivity (95%CI)	Specificity (95%CI)	Sensitivity HPV DNA	Specificity HPV DNA
Ref. 58	P16 cytology	ASC-US	385	92.6% (84.6–97.2)	63.2% (57.5–68.6)	90.1% (81.5–95.6)	37.8% (32.4–43.5)
		LSIL	425	92.0% (86.1–95.9)	37.1% (31.4–43.0)	95.7% (91.0–98.4)	18.5% (14.2–23.5)
Ref. 61	p16/Ki-67 cytology	ASC-US	361	92.2% (83.8–97.1)	80.6% (75.6–85.1)	90.9% (82.2–96.3)	36.3% (30.7–42.2)
		LSIL	415	94.2% (88.8–97.4)	68.0% (62.2–73.4)	96.4% (91.7–98.8)	19.1% (14.6–24.2)
Ref. 62	p16/Ki-67 cytology	HPV positive ASC-US	140	81.8% (63.9–92.4)	62.3% (52.3–71.3)	–	–
		LSIL	264	86.8% (77.7–92.7)	57,6% (49.8–65.0)	92.2% (84.1–96.5)	35.3% (28.3–42.9)
Ref. 63	p16/Ki-67 cytology	ASC-US+LSIL	256	89.7% (78.8–96.1)	73.1% (65.6–79.8)	96.6% (88.1–99.6)	68.1% (60.3–75.3)

HPV human papillomavirus, ASC-US atypical squamous cells of undetermined significance, LSIL low grade squamous intraepithelial lesion. CIN cervical intraepithelial neoplasia, CI confidence interval.

of all HPV positives was more than double (ratio *vs*. cytology 2.38: 2.21–2.57).

More recently other studies have applied the p16INK4a/Ki-67 dual staining technology. In a study conducted within a pilot project in Germany, 425 HPV positive cytology-negative women were tested for p16INK4a/Ki-67 dual staining.[69] These women were referred for repeat cytology after 6 months and repeat HPV/cytology after 12 months. Any positive cytology and/or HPV test during follow-up triggered colposcopy. Sensitivity for CIN2+ (91.9% 78.1–98.3) was similar to that obtained in the NTCC study with p16INK4a and that for CIN3+ (96.4%; 81.7–99.9) slightly higher. Specificity was higher than in NTCC: 82.1% (72.9–89.2) for <CIN2 and 76.9% (67.6–84.6) for <CIN3. It is difficult to define how much of this difference is due to the use of dual staining and how much to the underlying population (HPV-positive and cytology negative instead of all HPV-positive women).

Other methods have been applied for triaging HPV-positive women. Cytology is the most common. US guidelines[70] recommend co-testing with HPV and cytology, referring immediately to colposcopy HPV-positive women with abnormal cytology (ASC-US or more severe) and retesting HPV-positive cytological negative women after one year, with the option of sending HPV16 or HPV18 positive women to immediate colposcopy. Women positive at the 1-year repeat cotest for either test should be referred to colposcopy. The cross-sectional accuracy of cytology, genotyping and their combinations has been investigated in the ATHENA study[71] in a large population of women who were previously cytologically negative and who were cotested for cytology and HPV. With histologically determined CIN2+ as endpoint, sensitivity among HPV-positive women was 52.6% (47.6–57.6) for ASC-US or worse cytology, 51.8% (46.8–56.8) for HPV 16 or HPV 18 presence and 74.5% (69.9–78.6) for either cytology ASC-US+ or HPV 16 or HPV 18 presence. The percentage of HPV-positive women who were positive to each criterion was 27, 28 and 45% respectively.

The best interval after which HPV-positive women negative for a triage test should be retested is still an issue of ongoing debate. Because the time needed for progression from HSIL to invasion is estimated to be very long,[13,72] lesions may have already been existing for a long time, especially at the first screening with an HPV-test even in women previously screened by cytology. Indeed, the NTCC trial observed a significant difference in cancer incidence between the HPV and cytology group already within 3.5 years,[65] suggesting that some CIN3 had been repeatedly missed by cytology. Therefore, cross-sectional sensitivity is also a relevant consideration: cytology and HPV16/18 genotyping both have sensitivities below 80%, resulting in a high enough risk among test-negatives that warrants a one year repeat especially at the first screening round with HPV. The 90% sensitivity observed in most studies of p16INK4a results in a lower risk in test negatives, suggesting that the first repeat in HPV-positive, p16INK4a negative women could be done at longer intervals compared with those who are cyto-negative at triage. The longitudinal accuracy of p16INK4a immunostaining was studied in the NTCC trial[9] (Table 2). In that study, HPV-positive women were followed up with cytology and HPV testing: women were tested at one year interval as long as HPV remained positive and referred to colposcopy if cytology was ASCUS+. The p16INK4a result at baseline was strongly associated with the cumulative detection of CIN3+. Among women aged 35–60 years at recruitment the risk of developing a CIN3+ during 3 years of follow-up was 4.7% among HPV and p16INK4a-positive women compared with just 0.8% in HPV positive but p16INK4a negative women (relative risk 6.05; 1.38–26.5) and 83.7% of women who had a CIN3+ detected during this follow-up were p16INK4a-positive at baseline. Furthermore, no invasive cancer was detected either at baseline or during follow-up among p16INK4a-negative women. The association with CIN2 was lower (RR 2.11; 0.65–6.81) and the cumulative risk of CIN2 in p16INK4a-negative women was 1.7%. If just HPV and p16INK4a-positive women had been referred to colposcopy and had post-colposcopy

Table 2. Cross-sectional and longitudinal sensitivity and specificity of p16 or p16/ki67 in primary screening to identify CIN 2+ and CIN 3

Study	Test	Population	Sample size	Outcome	Sensitivity (95%CI)	Specificity (95%CI)
Ref. 8	p16 cytology	HPV-positive Cross sectional	1137	CIN 2+	88% (80–94)	61% (57–64)
				CIN 3	91% (77–97)	59% (55–63)
Ref. 9	p16 cytology	HPV-positive longitudinal	793	CIN 2+	66.9% (52.4–79.5)	NA
				CIN 3	77.8% (63.9–91.6)	NA
Ref. 69	p16/Ki-67 cytology	HPV-positive, cyto-negative	425	CIN 2+	91.9% (78.1–98.3)	82.1% (72.9–89.2)
				CIN 3	96.4% (81.7–99.9)	76.9% (67.6–84.6)
Ref. 73	p16/Ki-67 cytology	Primary screening	24577	CIN 2+	86.7% (81.1–90.9)	95.2% (94.9–95.4)
				CIN 3	87.4% (79.5–92.5)	94.8% (94.5–95.1)

CIN cervical intraepithelial neoplasia, CI confidence interval, HPV Human papillomavirus, NA non available.

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follow-up, the relative sensitivity for CIN3+ *versus* cytology during 3 years would have been 2.08 (1.13–3.56), similar to that (2.43; 1.46–4.04) obtained applying this protocol to alI HPV-positive women. In contrast, this approach would have reduced by over 50% the number of women who had this post-colposcopy follow-up and the number of those who had further colposcopies and biopsies during it.

Another way of choosing retesting intervals is comparing the cumulative detection of CIN3+ from recruitment observed in women who were HPV-positive but $p16^{INK4a}$-negative at baseline (2.0%) to that observed in women who were HPV-negative (0.01%)[9] (Table 2). The latter was clearly much lower, showing that $p16^{INK4a}$ is able to select among HPV-positive women a population at low risk but not as low as HPV-negatives. Thus, retesting in HPV-positive $p16^{INK4a}$-negative women must be at shorter interval than in HPV-negative women.

Considering all women who were cytological normal (and mostly also HPV-negative) at baseline, the 3-year cumulative incidence of CIN3+ (0.04%)[9] was again much lower than in HPV-positive $p16^{INK4a}$-negative women. However, no invasive cancer was observed among $p16^{INK4a}$-negative women, whereas 7/16,940 women with normal cytology at baseline had an invasive cancer detected at the subsequent screening round.[9] Therefore, it appears to be safe for HPV-positive $p16^{INK4a}$-negative women to have intervals similar to those applied to cytological normal women, which is 3 years in most European countries, but more data are needed to make specific recommendations.[7]

The $p16^{INK4a}$/Ki-67 dual staining in primary screening

The $p16^{INK4a}$/Ki-67 dual stain cytology was also tested in a very large cross-sectional clinical trial in five countries across Europe enrolling 27,349 women in a screening setting (Table 2). Pap, HPV (HC2) and $p16^{INK4a}$/Ki-67 dual-stained cytology testing were performed, and all women with any positive test result (except for HPV test positivity in women aged <30 years as the only positive test) were referred to colposcopy/biopsy.[73] The overall prevalence of positive dual-stained cytology test results was 5.4%, similar to the prevalence of ASC-US+ (5.2%), and half of the prevalence of HPV (10.7%); the $p16^{INK4a}$ /Ki-67 dual-stained cytology had significantly higher sensitivity for CIN2+ than Pap cytology (86.7 *vs.* 68.5%) and for CIN 3+ (87.4 *vs.* 73.6%) while maintaining comparable specificity (95.2 *vs.* 95.4%) and (94.8 *vs.* 95.1), irrespective of age. In women older than 30 years, HPV testing in this screening cohort was more sensitive for diagnosing CIN2+ and CIN3+ than the $p16^{INK4a}$/Ki-67 dual-stain (93.3 *vs.* 84.7% and 96.2% *vs.* 87.2%, respectively), but significantly less specific (93.0 *vs.* 96.2% and 92.7 *vs.* 95.9%, respectively). The results of this large cross-sectional study show that the dual $p16^{INK4a}$/Ki-67 cytology test offers a potential alternative to screen for HSIL, compensating the low sensitivity of cytology while maintaining its specificity. However, longitudinal data are needed in order to define screening intervals for the $p16^{INK4a}$/Ki-67 dual-stain cytology-negative women. The NTCC data mentioned above suggest more frequent screening is required when using dual stain cytology compared with HPV testing.

The combination of these novel staining techniques with computer-assisted imagine analysis is of course the next reasonable step of development. Initial feasibility studies have shown that the combinations of the $p16^{INK4a}$/Ki-67 with computer assisted microscopy yields an excellent sensitivity and an almost optimal specificity to detect women who have developed HSIL lesions.[74]

Conclusions

The increasing number of studies in which $p16^{INK4a}$ has been used as discriminating maker to highlight HPV-transformed cells in cervical specimens including both formalin fixed paraffin embedded biopsies and cytology samples strongly suggests that its clinical application will help to better unravel the real biology behind histological or cytological lesions. This is of particular relevance for the large group of equivocal changes that were up to recently hard to interpret. This will avoid substantial ambiguity in the pathologists' diagnoses and help to facilitate the pathologists reporting to the clinician. However, its implementation in clinical guidelines still awaits its realization on a broad internationally accepted scale and additional markers may be studied that could further help to more specifically predict the course of p16^{INK4a}-positive lesions, as not all of them progress. The latter will be an important task for the near future.

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