"Grounds for modification of algorithm for histological examination of the cervical tissue analysis"

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Resume

Nearly 100% of cervical dysplasia and cancers are associated with infection with high risk human papilloma viruses (HR HPVs). However, there are no "perfect" HR HPV tests for the tissues because HPV viral load is lower in FFPE than liquid cytology. For detection of early HPV proteins E5, E6 and E7 associated with tumorigenesis, there are no reliable antibodies able to detect antigen expression in tissues. HR-HPV may be episomal (hard to detect) or integrated (patchy signals), hence negative for presence of the whole genome and/or expression of viral proteins. Besides, detection of HR HPV does not immediately imply a disease. Over 90% of HR HPVs are cleared spontaneously within a year. Perception of positivity for HR HPV as a disease results in overtreatment and may end up in infertility. Clash between variable disease course and stringent treatment recommendations points at the acute need for new methods alternatively modification of the current methods which can effectively diagnose/prognose cervical pathology from the very early stages to guide/personalize the treatment. Proposed to be implemented for the diagnostics of CIN and CC in PSUKH are two modifications:

(1) A new testing algorithm with the use of combination of the markers p16 and Ki-67 with a marker not traditionally used for CIN/CC diagnosis, namely tumor suppressor protein p53, with interpretation of results of semi-quantitative assessment of expression of each marker, with.

(2) A new methodology implying transfer from to method of mono- stainings, to the dual and triple staining for cellular markers to allocate the levels of marker expression to one and the same cells, specifically important for p16 and p53.

Planned improvement of the algorithm for screening of cervical neoplastic lesions (CIN) and cervical cancer would answer the requirement of the Cabinet of Ministry of Latvia towards improvement of the cancer screening (Government of Latvia, Cabinet of Ministers, https://tapportals.mk.gov.lv ; Public Consultation Proposals for the Project "Healthcare Service Improvement Plan in Oncology for 2025-2027" https://tapportals.mk.gov.lv/attachments/legal_acts/additional_documents/e8fd27e8-eb7a-4b01-9e53-5f2643a83c7b/download)

The document is composed by Dr Jurijs Nazarovs, the Pathology institute, Paul Stradins Clinical University Hospital (PSUKH) and Dr Maria Isaguliants, Institute of Micribiology and Virology, Riga Stradins University, for further approval by the Chief Physician, and the Chief Oncologist of PSUKH, and approbation and acceptance by the Quality Department of PSUKH.

I. Standard Algorithm for Cervical Neoplastic Lesion Diagnostics via Histochemistry and Immunohistochemistry

I.1 Materials for analysis

Institute of Pathology of Paul Stradins Clinical University Hospital (PSUKH) receives various types of cervical tissue samples for diagnostic purposes, depending on the clinical scenario and the suspected pathology. These samples are collected and sent to the hospital for diagnostics by the gynecologist after abnormal Pap smears by liquid cytology, detection of HPV positivity for the high risk human papilloma viruses (HR HPVs) in Pap test, detection of visible lesions during colposcopy, or surgery.

The main types of cervical tissues submitted for diagnostics are:

- Biopsy Samples include; (i) Punch biopsy (small circular tissue samples taken from suspicious areas of the cervix, often during colposcopy); (ii) Cone biopsy or conization materials (cone-shaped tissue sample removed via LEEP (Loop Electrosurgical Excision Procedure) or cold-knife techniques, used for both diagnosis and treatment of high-grade lesions); (iii) Endocervical curettage (ECC) (scrapings from the endocervical canal to assess for glandular abnormalities).

- Surgical Specimens: (i) LEEP/LLETZ specimens (larger excisional samples from procedures like LEEP, used to remove and examine precancerous or early cancerous lesions); (ii) Hysterectomy specimens (the entire cervix (and uterus) may be examined in cases of confirmed cancer or extensive dysplasia).

Tissues received for analysis, are fixed in formalin, processed for histopathology (H&E staining), and undergo ancillary tests, first by histochemistry followed by immunohistochemistry (IHC) for selected set of human markers.

I.2 Histochemical Characteristics for Diagnostics of CIN and Cervical Cancer in H&E-Stained Slides

Cervical tissues are first analysed by histochemistry. The diagnosis of cervical intraepithelial neoplasia (CIN) and cervical cancer relies on distinct histomorphological features observed in H&E-stained slides. The key diagnostic criteria are listed below.

I.2.1 Normal Cervical Epithelium (Baseline for Comparison)

Stratified squamous epithelium is characterized by: (i) Basal/parabasal layers: Small, uniform nuclei, high nuclear-to-cytoplasmic (N:C) ratio; (ii) Intermediate/superficial layers: Maturation with glycogen-rich cytoplasm (clear/pink).

I.2.2 Endocervical glandular epithelium: Single layer of columnar cells with mucin-filled cytoplasm.

I.2.3 Cervical Intraepithelial Neoplasia (CIN)

CIN1 (LSIL) – Low-Grade Squamous Intraepithelial Lesion: (i) Koilocytosis (hallmark of HPV infection): (ii) Perinuclear halos (clearing due to HPV cytopathic effect); (iii) Nuclear enlargement, hyperchromasia, irregular contours. Lesions are confined to lower 1/3 of epithelium. Mitoses rare, limited to basal layer.

CIN2 (HSIL) – High-Grade Squamous Intraepithelial Lesion, Moderate Dysplasia); (i) Atypia extends to middle 1/3–2/3 of epithelium; (ii) Loss of maturation (immature basaloid cells dominate); (iii) Increased mitoses (may include abnormal forms); (iv) Nuclear pleomorphism (variation in size/shape).

CIN3 (HSIL) – Severe Dysplasia/Carcinoma *In Situ*: (i) Full-thickness atypia (no surface maturation); (ii) Crowded, hyperchromatic nuclei with high N:C ratio; (iii) Frequent mitoses (including upper layers); (iv) Architectural disarray (loss of polarity).

I.2.4 Invasive Squamous Cell Carcinoma (SCC)

SCC is characterized by stromal invasion: (i) Irregular nests/clusters of malignant cells breaking through basement membrane; (ii) Desmoplastic reaction (fibrotic stroma around tumor); (iii) Cytologic atypia, including prominent nucleoli, coarse chromatin; (iv) Keratin pearls (well-differentiated SCC) or anaplastic cells (poorly differentiated).

I.2.5 Adenocarcinoma (Endocervical Type) (ACet)

ACet is characterized by: (i) Glandular architecture with cribriform/papillary patterns; (ii) Nuclear stratification, mitotic activity; (iii) Mucin depletion (vs. normal endocervix); (iv) Stromal invasion (single cells or irregular glands).

Their descriptive qualitative and quantitative characteristics in the proposed protocol remain unchanged, and includes definition of the following parameters:

1. The presence or absence of dysplasia;

• if present - an indication of the degree of dysplasia (CIN I, CIN II, CIN III).

2. The presence or absence of a malignant neoplasm;

• if present - determination of the histological type of tumor;

• an indication of the degree of differentiation (Grade).

3. Evaluation of concomitant morphological changes - inflammatory, cicatricial, etc.).

II. Ancillary analysis for confirmation of CIN/CC diagnosis

The diagnosis of cervical intraepithelial neoplasia (CIN) and cervical cancer based on the distinct histomorphological features observed in H&E-stained slides is further supplemented by immunohistochemistry (IHC). Nearly 100% of CIN and cancer cases are associated with infection with HR HPVs, >70% with infection with HPV16 and HPV18 (https://www.cancer.gov/types/cervical/causes-risk-prevention). Two alternative diagnostic traits are: (1) detect in tissues the virus(es) (HR HPVs); (2) detect changed caused by the virus.

II. 1 Direct analysis for HR HPVs

Challenges in HR HPV detection in tissues are multiple. Firstly, HPV is episomal (non-integrated) in most precancers, making viral DNA/RNA detection harder than in cervical swabs. Secondly, formalin-fixed paraffin-embedded (FFPE) tissues degrade nucleic acids, reducing sensitivity. Third, there is no single "gold standard" test which would balance affordability, ease, and reliability perfectly. Pros and cons of the available methods are listed in Table 1.

Table 1. Pros and cons of available methods of detection of HR HPVs in tissues. Surrogate host marker p16 is given for comparison * Cost interpretation: $ - affordable; $$ $$$ - extremely expensive

Method	Pros	Cons	Complexity	Cost*
Surrogate marker **p16 IHC** (surrogate)	Cheap, easy, widely available.	Indirect (not HPV-specific); false +/-.	Low	$
HPV DNA ISH (CISH/SISH)	Visualizes HPV in nuclei.	Low sensitivity (30–70%); expensive.	Medium	$$$
PCR (DNA/RNA)	Highly sensitive/specific.	Requires DNA extraction, la infrastructure.	High	$$
RT-PCR (E6/E7 mRNA	Best for active infection.	Complex, needs fresh tissue/RNA.	Very High	$$$$
NGS (WGS/targeted)	Detects HPV type + integration.	Expensive, specialized labs only.	Very High	$$$$$

No "perfect" HR HPV tests exist because HPV viral load is lower in FFPE than liquid cytology. For detection of early HPV proteins E5, E6 and E7 associated with tumorigenesis, there are no reliable antibodies able to detect antigen expression in tissues. HR-HPV may be episomal (hard to detect) or integrated (patchy signals), hence negative for presence of the whole genome and/or expression of viral proteins.

There are no straightforward inexpensive technologically uncomplicated tests to detect HR HPV in tissues.

Furthermore, analysis for HR HPV DNA only can be misleading. Guided by the paradigm that there are no effective methods to clear HPV infection once established, the American Cancer Association screening guidelines advocate for “screen-and-treat” and “screen-triage-treatment” strategies (*Xu L Gynecol Obstet Clin Med 2023*). HPV(+) CIN, specifically CIN2/3 cases, are considered precancerous, and request ablative or excisional treatment. This is often unnecessary, since 60% of HPV infections are cleared within months and 90%, within a year after detection (*Bulkmans NW Br J Cancer 2007; Huber J. Womens Health (Lond). 2021*), i.e. do not need treatment, while CIN treatment may cause reproductive health problems (*Papoutsis D BJOG 2012; Kyrgiou M BMJ 2014*).

To conclude, "simple & cheap" HPV tissue tests do not yet exist, though research is ongoing, and results of HR HPV tests alone are insufficient for both diagnostics and treatment choice.

II.2 Host markers associated with HR HPV infection and their selection

Diagnostic laboratory, also in PSUCH, uses detection of host molecular markers, i.e. not the virus itself, but changes induced by HR HPV. These markers help identify high-risk HPV activity, grade lesions, and predict progression risk. The key markers and their clinical utility are described below, additional markers, in Table 2.

p16^{INK4a} detects the overexpression of the p16 protein, a tumor suppressor upregulated when HR HPV oncoprotein E7disrupts the retinoblastoma (Rb) pathway. This is a Gold standard surrogate for HR HPV activity (e.g., HPV-16/18). It distinguishes HSIL (CIN2/3) from benign/reactive changes (diffuse strong staining = HSIL), and Resolves ambiguous morphology (e.g., mimics of CIN like atrophy).

Ki-67 (Proliferation Marker) detects nuclear protein expressed in proliferating cells. Marker Grades cervical intraepithelial neoplasia (CIN) as: (i) LSIL (CIN1) when staining is limited to lower 1/3 of epithelium; (ii) HSIL (CIN2/3) in case of the full-thickness proliferation (upper epithelial layers).

Other IHC markers: (1) ProEx C (TOP2A/MCM2) for the aberrant S-phase cell cycle proteins (TOP2A, MCM2) induced by HPV E6/E7 sensitive for HSIL and early adenocarcinoma, which helps to identify false-negative p16 cases (e.g., some hrHPV+ lesions with weak p16); (2) HPV L1 Capsid Protein - late HPV protein expressed in productive infections (transient HPV). L1+ lesions (common in LSIL) often regress spontaneously, staining is negative in HSIL/cancer which indicates integration of HPV DNA with the loss of parts of HR HPV genome encoding late/capsid proteins; (3) CDKN2A (p16 Gene) Methylation detects epigenetic silencing of *p16* gene with hypermethylation correlating with cancer progression; (4) Insulin-like Growth Factor mRNA-Binding Protein (IMP3) - oncofetal protein overexpressed in HSIL/cancer, distinguishes CIN2 likely to progress (Table 2).

Table 2. Utility of basic markers for diagnostics of CIN and CC

Marker	Utility
p16^{INK4a}	Diffuse strong staining → HSIL/cancer (HPV-driven).
Ki-67	High proliferation (full-thickness in HSIL).
p63/p40	Confirms squamous differentiation (SCC).
CEA	Highlights glandular lesions (for adenocarcinomas).
ProEx C (TOP2A/MCM2)	To identify false-negative p16 cases (for adenocarcinomas).
HPV L1 Capsid Protein	Positive staining associates with spontaneous regression
CDKN2A (p16 Gene) Methylation	Detects hypermethylation correlating with cancer progression

Use of indirect markers help to avoid the limitations of HR HPV DNA testing, and evaluate the down-stream effects of HR HPV infection – i.e. the biologic impact of HR HPVs, not just presence.

III. Tumor suppressor protein p53 as a marker for CIN and CC

The p53 protein, often called the "guardian of the genome," acts as a tumor suppressor by regulating cell division and maintaining genetic stability. It's a transcription factor that binds to DNA and controls the expression of various genes involved in cell cycle arrest, DNA repair, and apoptosis (programmed cell death). Essentially, p53 helps prevent cells from growing and dividing uncontrollably, which could lead to cancer (https://medlineplus.gov/genetics/gene/tp53). Traditional use of p53 as IHC marker is summarized in Table 3.

In the frame of research done on LZP project 202/1-0484, we have detected the wild-type (normal) heterogeneous staining in the normal epithelium, but also in LSIL (CIN1) and most HSIL (CIN2/3). Furthermore, we detected cases of complete absence of p53 on the background of absence of p16, and overexpression of 53 (mutant) on the background of high expression of p16. The first speaks for suppression of expression of p53 unrelated to HR HPV infection (since tissues are p16 negative), i.e. suppressionnot

caused by the activity of HR HPV oncoprotein E6. This indicates mutations in p53 causing its loss, and hence loss of control of tumor suppression. The second speaks for p53 expression despite presence of HR HPV which by activity of oncoprotein E6 should have targeted p53 to proteasome, i.e. presence of mutant non-degradable and hence inactive form of p53, which also means the loss of control of tumor suppression.

Table 3. ICH staining for tumor suppressor protein p53 with interpretation of the results

Pattern	Interpretation	Associated Lesions
Wild-type (normal)	Scattered nuclear positivity in basal cel (heterogeneous staining).	Normal epithelium. LSIL (CIN1), most HSIL (CIN2/3).
Overexpression (muta	Strong, diffuse nuclear staining in >70% cells (aberrant pattern).	Unrelated gastric-type adenocarcinom some SCCs.
Complete absence	Null pattern (no staining, due to nonsen mutations or deletions).	**HPV-independent cancers** (e.g., ader basal carcinoma).
Cytoplasmic staining	Non-specific; may indicate p53 dysfunc or technical artifact.	Inconclusive; requires correlation.

Thus, both detection of strong diffuse p53+ + p16+ and null p53 + p16- points at CIN and CC having driving factors additional to HR HPVs, requesting analysis of genetic background of the patient.

IV. Clinical workflow using combination of HR HPV associated host markers

1. **H&E morphology** → standard, as described above
2. **p16**: **p16** : → suspicion for HR HPV associated HSIL (manage aggressively); **p16** → Likely benign.
3. **Ki-67**: **Ki-67+** → suspicion for HSIL (manage aggressively); **Ki-67–** → Likely benign.
4. **Combination of the resutls (today)**
    - **p16+/Ki-67+** → HSIL (manage aggressively).
    - **p16–/Ki-67–** → Likely benign.
5. **Proposed p16/Ki-67 simultaneous staining for both markers (require new test systems)**
    - **p16+/Ki-67+** → HSIL (manage aggressively).
    - **p16–/Ki-67–** → Likely benign.
6. ***TP53*/P53 complement to p16 and Ki-67 stainings, alternatively triple p16/Ki-67/TP53:**
    - **p16+/Ki-67+/TP53 overexpression** → HSIL (manage aggressively), revert to genetic tests
    - **p16–/Ki-67+/TP53 overexpression** → HSIL (manage aggressively), revert to genetic test.
    - **p16–/Ki-67+/TP53 null** → Aggressive HPV-negative cancer, revert to genetic test

- **p16−/Ki-67−/TP53wt** → Likely benign.
- **p16−/Ki-67+/TP53wt** → Closer surveillance should be considered.

The material basis and all necessary equipment for dual p16/Ki-67 staining is available: fluorescent microscope Ecliplse Ci-Lplus (Nikon) installed in 2024, Autostainer Link 48, Coverstainer, CS100 Coverslipper (Dako Pathology, Agilent) to perform advanced immunochemical analysis planned in the project. Ecliplse Ci-Lplus has DAPI-FITC-TRITC filter combination allowing simultaneous detection of cell nucleus (DAPI) and 3 biomarkers.

V. Expected Effect

The use of host-marker combination will personalized time line of the follow ups and approaches to treatment. Specifically, the combined Ki-67/**p16** would improve the diagnostic accuracy, e.g., p16+/Ki-67+ indicates HSIL cases, and addition of p53 marker, specifically performance of ., p16/Ki-67/p53 triple tests will allow to distinguish aggravated cases of HR HPV+ CIN and CC on the background of p53 mutations, as levels of expression of p16 and p53 could be allocated to one and the same cell. Marker combinations will offer possibility to make an educated choice between observation, surgical excision, and p53 patterns would guide for the extended analysis of the genetic background of the patient to identify genetic predisposition to cancer development. Genetic tests are recommended to rule out rare subtypes. Additional IHC testing should be requested (ER/PR, HER2) to exclude to exclude gastric-type adenocarcinoma (p53+, p16−), and adenoid basal carcinoma (p53−). Closer surveillance should be considered.

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