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Pitfalls in Gynecological Cytology: Review of the Common and Less Frequent Entities in Pap Test

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Gynecological cytology · Pap test · Pitfalls

Abstract
Background: Pitfalls in Pap test could be defined as false positive, false negative, or underdiagnosed results which can lead to unnecessary diagnostic procedures or delayed and inadequate treatment. It can be a consequence of misinterpretation of certain morphological entities which are described in this paper. ***Summary:*** The paper presents an overview of the morphological features and look-alikes of the common sources of pitfalls such as atrophy, repair, intrauterine device change, tubal metaplasia, hyperchromatic crowded groups, and radiation changes. Rare causes of pitfalls such as Arias-Stella changes, pemphigus, tumor diathesis per se, rare types of cervical cancer, including verrucous and papillary squamous cell cancer, gastric type, and endometrioid adenocarcinoma are also described. ***Key Messages:*** The awareness of pitfalls in cervical cytology is important for cytopathologists and clinicians to avoid future errors. Review of Pap tests with erroneous diagnosis is important for quality control in cytology laboratory, and it must be considered an educational- and experience-building procedure. Cytopathologist should not pull back in significant diagnoses, especially in human papillomavirus-negative cases.

Introduction

Cervical cancer (CC) screening is a population-based mass testing of women and people with cervix with the purpose of prevention of invasive uterine CC. Different policies are used in CC screening in different countries worldwide. In more than 70% of the countries, cytology is still used as a primary test in CC screening, followed by primary human papillomavirus (HPV) screening and co-testing (simultaneous cytology and HPV testing) [1]. The sensitivity of cytology in the detection of precancer and early cancer is lower, compared to the HPV testing, while the co-testing outperforms the standalone testing, cytology, or HPV, having the highest sensitivity [2, 3]. Cervical cytology, or Pap test, is a diagnostic morphological test which analyses cellular characteristics

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under the light microscope, and its accuracy depends on the test performance. Following established morphological criteria, it can recognize the cellular substrate of premalignant and malignant cervical lesions resulting in early diagnosis and efficient treatment. However, in some cases, the Pap test result may be erroneous for several reasons such as inadequate sampling, poor technical processing, or interpretation pitfalls [4]. Pitfalls or errors of the Pap test could be false negative or false positive result, as well as reports of overdiagnosis or underdiagnosis of the lesion [5, 6]. In pathology, errors can be divided into major and minor. Major errors have a negative impact on the patient therapy, prognosis, and outcome of the disease. Minor error does not have a significant impact on the patient therapy, prognosis, and outcome [7]. A false positive Pap test means that a cytological diagnosis of a high-grade lesion or cancer is incorrectly given to the patient without disease, leading to unnecessary workup and biopsies. It can occur in certain clinical circumstances, and it is usually caused by misinterpretation of specific morphological entities. False positive Pap tests should be considered minor errors because they require histology confirmation and rarely lead to overtreatment. On the other hand, cytopathologists must be aware of their responsibility because false positive results can cause unnecessary psychological distress in patients [8, 9]. Major errors in Pap test could be defined as false negative or underdiagnosed results which can lead to delayed or inadequate treatment. This paper will give an overview of more and less common pitfalls in cervical cytology. Most descriptions are based on both conventional smears (CS) and liquid-based cytology (LBC) preparations because the morphological characteristics of described entities are similar in both methods. The cells and clusters in CS can appear larger and flatter due to smearing and air-drying and in LBC may appear smaller and rounded [4]. The purpose of this paper is to point out the look-alikes and difficulties in the interpretation of Pap test and to describe cytological characteristics that can help in rendering a correct and timely diagnosis.

Atrophic Pattern

Atrophy in cervical cytology is found in postmenopausal and postpartum women with a lack of estrogen and progesterone production. Multilayered squamous epithelium in atrophy consists only of basal, parabasal, or deep intermediate layers, without maturation to upper intermediate and superficial layers. In cytology, the squamous cells are smaller, often with increased nuclear-cytoplasmic (N/C) ratio presenting as single cells or in syncytial clusters or fragments, predominantly monolayered. The polarity of the nuclei in the cluster is regular with the longer axis of nuclei being parallel [4]. Nuclei are uniform, oval, with smooth borders and fine chromatin, without nucleoli. Atrophic patterns in the Pap test sometimes can pose a challenge in morphological interpretation. Occasionally, enlarged squamous cells with atypical nuclei and increased N/C ratio can be observed. These nuclei can be slightly hyperchromatic leading to the false diagnosis of atypical squamous cells (ASCs) or even high-grade intraepithelial lesion (HSIL) [6, 10, 11]. The main features to discriminate benign atrophy change and HSIL are chromatin distribution and nuclear borders (shown in Fig. 1a, b). Another feature of atrophic epithelium can lead to false positive results. Deep basal layers desquamated in sheets and fragments can present as hyperchromatic crowded groups (HCGs), showing nuclear hyperchromasia and overlapping. In atrophy, nuclear polarity is preserved (shown in Fig. 2a). In HSIL presenting as HCG (shown in Fig. 2b), nuclear polarity is disturbed with a longer axis of single nuclei pointing in criss-cross directions. Additional immunocytochemical dual staining with p16/Ki-67 can help, being positive in HSIL (shown in Fig. 2c).

Overview of morphological features of atrophy and HSIL is presented in Table 1. The background in atrophy is sometimes inflammatory with cellular debris which can be misinterpreted as tumor diathesis. Globular basophilic material known as the "blue blobs" represent degenerative cellular changes and should not be mistaken for atypical or malignant naked nuclei [6].

False negative Pap test in the background of atrophy can occur in CC cases. Malignant cells can be overlooked for being small, slightly atypical, sometimes fiber shaped, often with size and shape of surrounding normal squamous parabasal cells. Nuclei can appear slightly irregular, sometimes elongated, with inconsistently observed coarse chromatin, often normochromatic. Degenerative, weakly stained cells with atypical shapes resembling fiber and tadpole cells can give impression of the "ghost" cells (shown in Fig. 3a, b). In The Bethesda System for Reporting Cervical Cytology was pointed out that atrophy must be carefully screened and analyzed to avoid such pitfalls [4]. To minimize the possibility of false negative reports in CC it is of utmost importance to have the data of clinical gynecological examination which can describe abnormal bleeding or cervical mass.

Repair

Reparative cellular changes or repair occurs as a reactive process in inflammation or epithelial damage of squamous or glandular epithelium. Reparatory cells can look dramatic

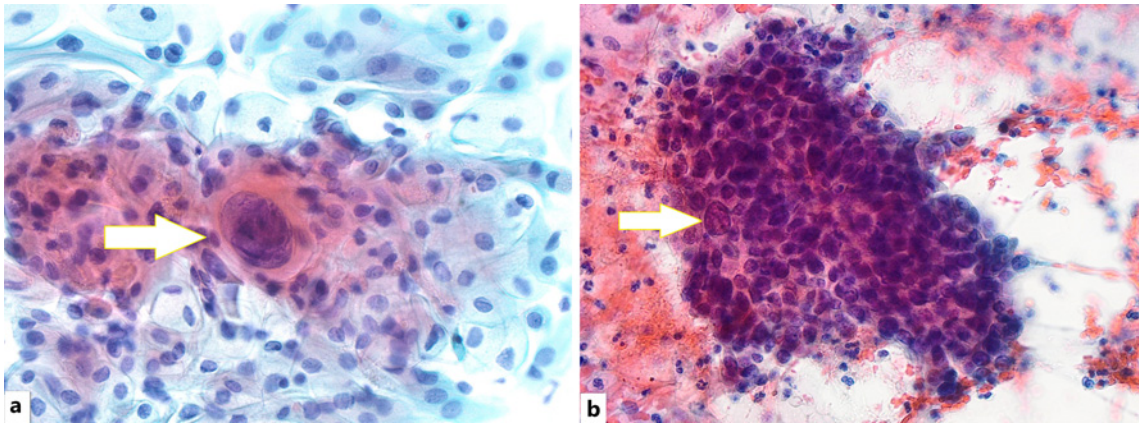

Fig. 1. a Atrophic change showing a single, enlarged squamous cell with a large nucleus, slightly hyperchromatic, with fine, partially smudged chromatin and regular nuclear border. Papanicolaou, ×400. **b** HSIL, dense cluster of ASCs with intense nuclear hyperchromasia, coarsely granular chromatin, irregular nuclear borders with indentations. Nuclei are predominantly of the same size with one extra-large atypical nucleus (arrow). Papanicolaou, ×400. HSIL diagnosis was confirmed by histology.

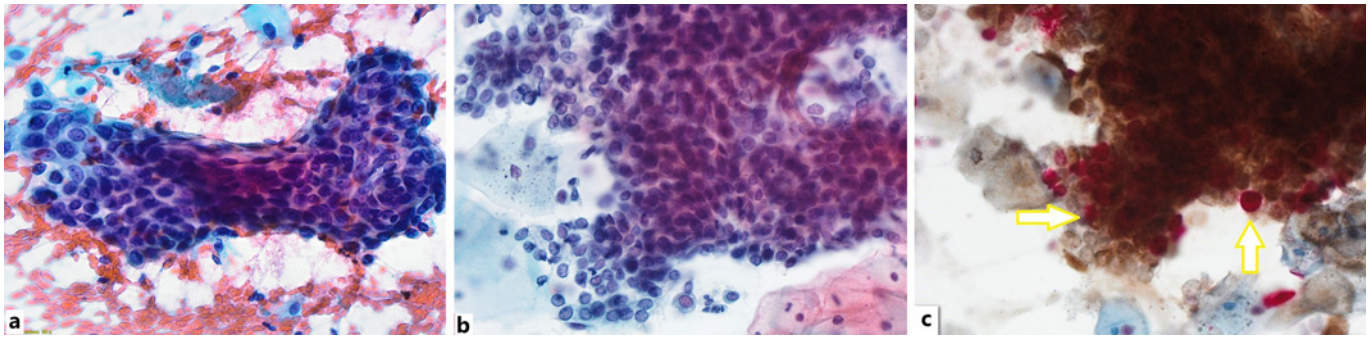

Fig. 2. a Atrophic epithelium presenting as HCG with nuclear hyperchromasia and overlapping. Chromatin is smooth, nuclear contours are regular, and nuclear polarity is preserved. Papanicolaou, ×400. **b** HSIL presenting as HCG, nuclear polarity is disturbed, chromatin is coarse, and nuclear borders are slightly irregular or indented. Papanicolaou, ×400. **c** HSIL with positive dual staining with p16/Ki-67 with brown cytoplasm (p16 signal) and red nuclei (Ki-67 signal) in the same cells. p16/Ki-67 immunostaining, ×400. HSIL diagnosis was confirmed by histology.

with the background of blood, leucocytes, and fibrin, which can give the impression of tumor diathesis. The main differentials of repair are HSIL and cancer [4, 5, 12–14]. Morphological features of repair (shown in Fig. 4a–c), HSIL, and cancer are shown in Table 2. In HSIL the nuclei are irregular and dark, nucleoli are inconspicuous, clusters are usually three-dimensional with disturbed polarity in opposition to repair which is presented in two-dimensional clusters, with preserved polarity and cytoplasmic borders, nuclei showing smooth chromatin and large nucleoli. In cancer, nuclei have irregular borders, nucleoli are irregularly shaped, and chromatin is clumped, alternating with "empty," light areas (parachromatin) (shown in Fig. 4d). Cell dispersion, disturbed polarity, dense hypercellular clusters are observed in squamous and poorly differentiated cancer, and in adenocarcinoma, typical glandular or papillary groups are seen. Intracytoplasmic neutrophils (so-called "bag of polys") can be prominent in repair, but also is frequently seen in adenocarcinoma cases, especially of endometrial origin. When observed, cannot be used to rule out malignancy [4]. It has been reported that one quarter of false negative adenocarcinoma in situ (AIS) cases are interpreted as reparatory change [15]. In some cases, the reparatory epithelium can show a much higher degree of atypia and is called atypical repair. It reflects in nuclear features such as prominent anisonucleosis, clumped chromatin, and irregular nucleoli, which overlap with characteristics of malignant cells [16]. In those cases, it is prudent to recommend colposcopy and biopsy to exclude underlying severe lesions.

Table 1. Atrophy and HSIL – morphological characteristics and p16/Ki-67 immunocytochemistry

	Atrophy with large, atypical nuclei	HSIL	Atrophy with HCG pattern	HSIL with HCG pattern
Nuclear size	Single or few enlarged nuclei	Usually enlarged	Small	Intermediate or small
Nuclear size variation	Present	Present	Absent	Absent or mild
N/C ratio	Moderate to high	High	High	High
Nuclear polarity	Preserved	Disturbed	Preserved	Disturbed
Chromatin	Fine, smooth, dense, or smudged	Coarsely granular, regular	Fine, smooth	Coarse, regular
Nuclear chromasia	Usually normochromatic can be slightly hyperchromatic	Hyperchromatic	Hyperchromatic	Hyperchromatic
Nuclear border	Smooth	Irregular, indented, convoluted	Regular	Irregular, indented, convoluted
Nucleoli	Absent	Absent	Absent	Absent
p16/Ki-67	Negative	Positive	Negative	Positive

HSIL, high-grade intraepithelial lesion; HCG, hyperchromatic crowded groups; N/C ratio, nucleo-cytoplasmic ratio.

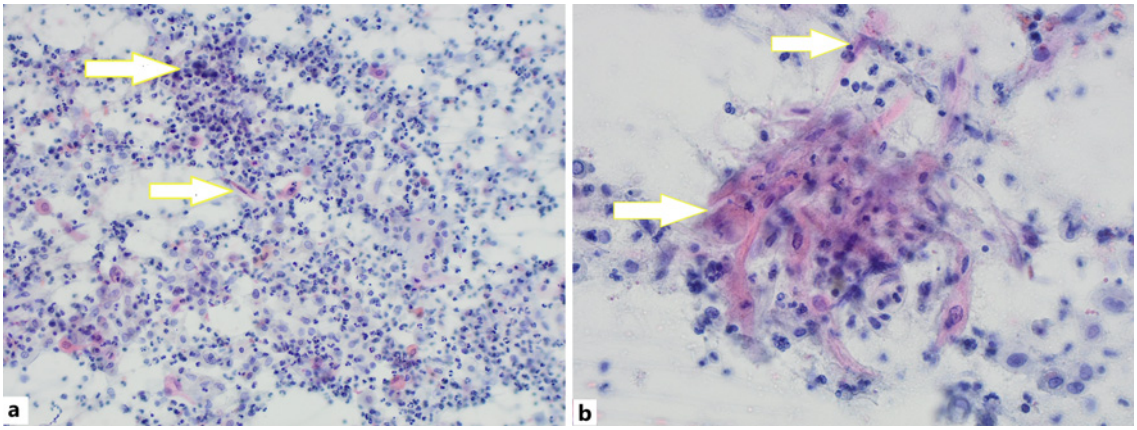

Fig. 3. a SCC in the background of atrophy with few atypical small, fiber-shaped, squamous cells (arrows). Papanicolaou, ×200. **b** SCC with slightly irregular, elongated, normochromatic nuclei, with inconsistent coarse chromatin. Degenerative, weakly stained "ghost" cells with atypical shapes resembling fiber and tadpole cells (arrows). Papanicolaou, ×400. SCC was confirmed by histology.

Intrauterine Device Atypia

The effect of foreign body irritation, such as intrauterine device (IUD), can sometimes cause reactive morphological changes in epithelial cells lining the endometrium and endocervix. These cells are called IUD atypia or IUD change. Table 3 Represents morphological characteristics of IUD atypia and its mimics. The cells can be shed as single cells, or in small glandular clusters with clean backgrounds. Nuclei are usually dark, often with dense or wrinkled chromatin. Nucleoli can be prominent. Cytoplasm can show large vacuolization giving the appearance of "signet ring" cells (shown in Fig. 5 a, b). Pseudo psammoma-bodies calcifications can be present in some cases. The main differential diagnosis is HSIL, sometimes adenocarcinoma [4, 5]. The main feature for discrimination is chromatin quality, which will be coarse and granular in HSIL (shown in Fig. 5c). In adenocarcinoma, nucleoli in combination with fine or

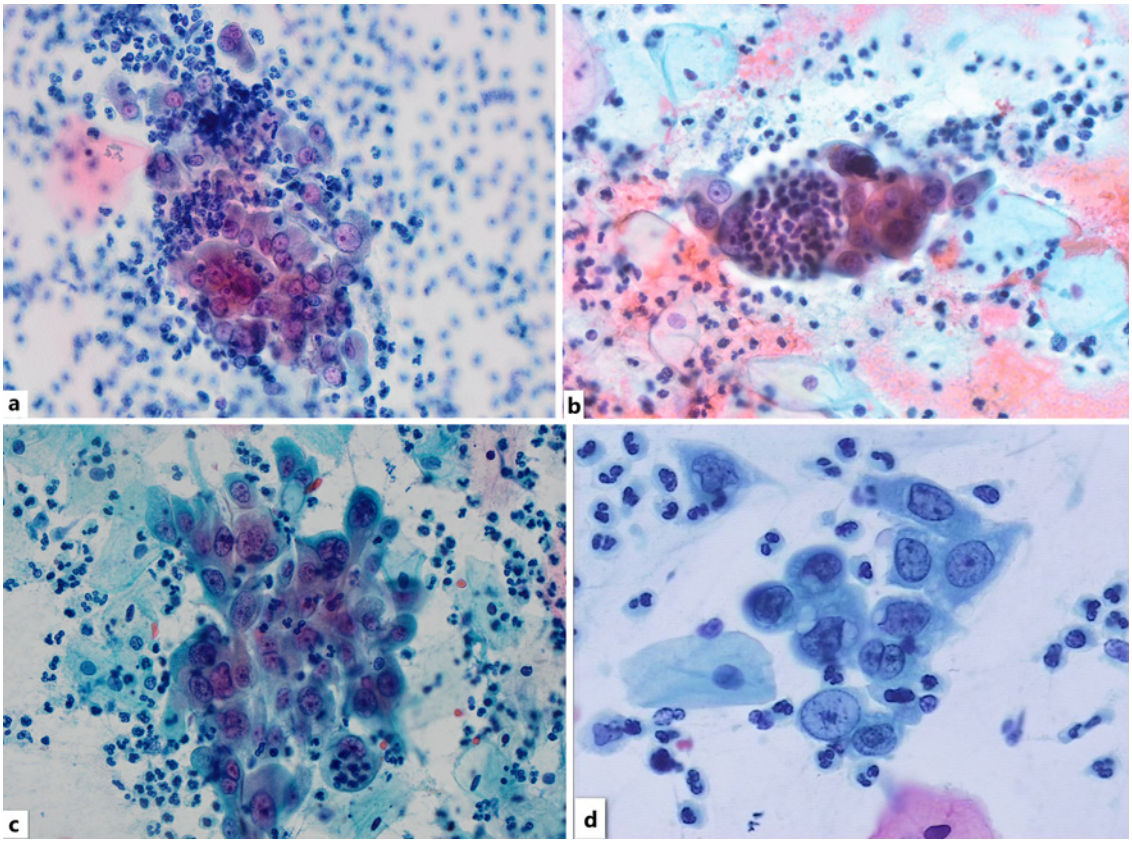

Fig. 4. a Reparatory epithelium showing anisonucleosis, anisocytosis, increased N/C ratio, smooth, reticulate chromatin, large single nucleoli, regular, thickened nuclear border. Papanicolaou, ×400. **b** Reparatory epithelium with well-defined cytoplasmic outlines, cytoplasm shows vacuolization, intracytoplasmic neutrophils, and polychromasia. Papanicolaou, ×400. **c** Reparatory epithelium with typical two-dimensional clusters, without overlapping, with preserved nuclear polarity and cellular borders. Papanicolaou, ×400. **d** Poorly differentiated squamous cell carcinoma with irregular nuclei and nucleoli, chromatin is clumped, alternating with "empty" areas (parachromatin). Papanicolaou, ×400. Benign reparative changes were confirmed by negative follow-up Pap tests, and SCC was confirmed by histology.

irregular chromatin can be seen in opposition to IUD atypia (shown in Fig. 5d). Knowledge of the patient clinical data about contraception is important in solving those cases.

Tubal Metaplasia

Tubal metaplasia is the process of metaplastic change of the endocervical glandular cells to the fallopian tube type of epithelial cells. It can present as pseudostratified HCGs of various sizes and shapes, like solid clusters, stripes, and rosettes, giving rise to the misdiagnosis of adenocarcinoma in situ (AIS) or HSIL. The cells in tubal metaplasia are enlarged, with a high N/C ratio, nuclei are hyperchromatic, can be slightly irregular, and usually are oval or elongated. Chromatin is evenly distributed, and nucleoli are inconspicuous. The cytoplasm is scant but can be vacuolated. The main feature that can help in discrimination from the high-grade lesion is a ciliary border, or terminal plates without cilia, which can be found after careful observation, sometimes only in the one edge or area of the cluster (shown in Fig. 6a–c) [4, 5, 17]. HPV test or dual stain with p16/Ki-67 can be helpful, being negative in tubal metaplasia (shown in Fig. 6d), and positive in AIS (shown in Fig. 6e, f).

Radiation Changes

Radiation changes occur mainly in squamous epithelium, after the radiotherapy of the pelvis area. Squamous cells can significantly be enlarged, affecting cytoplasm and nuclei, without an increased N/C ratio. The cells can be multinucleated with bizarre shapes. Chromatin is smooth, the nuclear border regular, and nucleoli are inconspicuous.

Table 2. Repair, HSIL and cancer – morphological characteristics

	Repair	HSIL	Squamous cell carcinoma	Adenocarcinoma
Nuclear size	Enlarged	Enlarged	Variable, usually enlarged	Variable, usually enlarged
Nuclear size variation	Mild to moderate	Present	Present	Variable
N/C ratio	Increased	Increased	Variable	Variable, usually increased
Nuclear polarity	Preserved	Disturbed	Disturbed	Disturbed
Chromatin	Smooth, regular, or reticulate	Coarsely granular, regular	Dark or clumped, alternating with "empty," light areas	Finelly granular
Nuclear chromasia	Usually normochromatic	Hyperchromatic	Variable, usually hyperchromatic	Normochromatic or mildly hyperchromatic
Nuclear border	Regular, thickened	Irregular, indented, convoluted	Irregular	Regular
Nucleoli	Present, large single, or multiple	Absent	Variable, irregular when present	Present, single or multiple
Cell shape	Usually polygonal	Round or polygonal	Variable, polygonal, round, elongated (fiber cells), bizarre shapes	Columnar, round, or oval with eccentric nuclei
Cytoplasmic features	Vacuolization, polychromasia	Dense, rarely vacuolised	Variable, keratinized, or basophilic	Basophilic, fine, sometimes vacuolised
Intracytoplasmic neutrophils	Present	Absent	Absent	Can be present in endometrial adenocarcinoma
Cytoplasmic borders in clusters	Preserved	Absent	Absent	Variable
Type of clusters	Two-dimensional clusters, without overlapping	Single cells, rows, three-dimensional in HCG	Single, dispersed, three-dimensional	Three-dimensional balls, rosettes, papillary

HSIL, high-grade intraepithelial lesion; HCG, hyperchromatic crowded groups; N/C ratio, nucleo-cytoplasmic ratio

Table 3. IUD atypia, HSIL, and adenocarcinoma – morphological characteristics

	IUD atypia	HSIL	Adenocarcinoma
Nuclear size	Enlarged	Enlarged	Variable, usually enlarged
Nuclear size variation	Mild to moderate	Present	Variable
N/C ratio	Increased	Increased	Variable, usually increased
Chromatin	Dense, wrinkled	Coarsely granular, regular	Finely granular or irregular
Nuclear chromasia	Hyperchromatic	Hyperchromatic	Normochromatic or mildly hyperchromatic
Nuclear border	Regular or irregular	Irregular, indented, convoluted	Regular
Nucleoli	Can be present	Absent	Present, single, or multiple

Table 3 (continued)

	IUD atypia	HSIL	Adenocarcinoma
Cell shape	Usually round to oval	Round or polygonal	Columnar, round, or oval with eccentric nuclei
Cytoplasmic features	Vacuolization, dense cytoplasm	Dense, rarely vacuolised	Basophilic, fine, sometimes vacuolised
Type of desquamation	Single cells, small glandular clusters	Single cells, rows, three-dimensional clusters	Three-dimensional balls, rosettes, papillary, glandular clusters
Background	Clean	Clean	Usually with blood and tumor diathesis

IUD, intrauterine device; HSIL, high-grade intraepithelial lesion; N/C ratio, nucleo-cytoplasmic ratio.

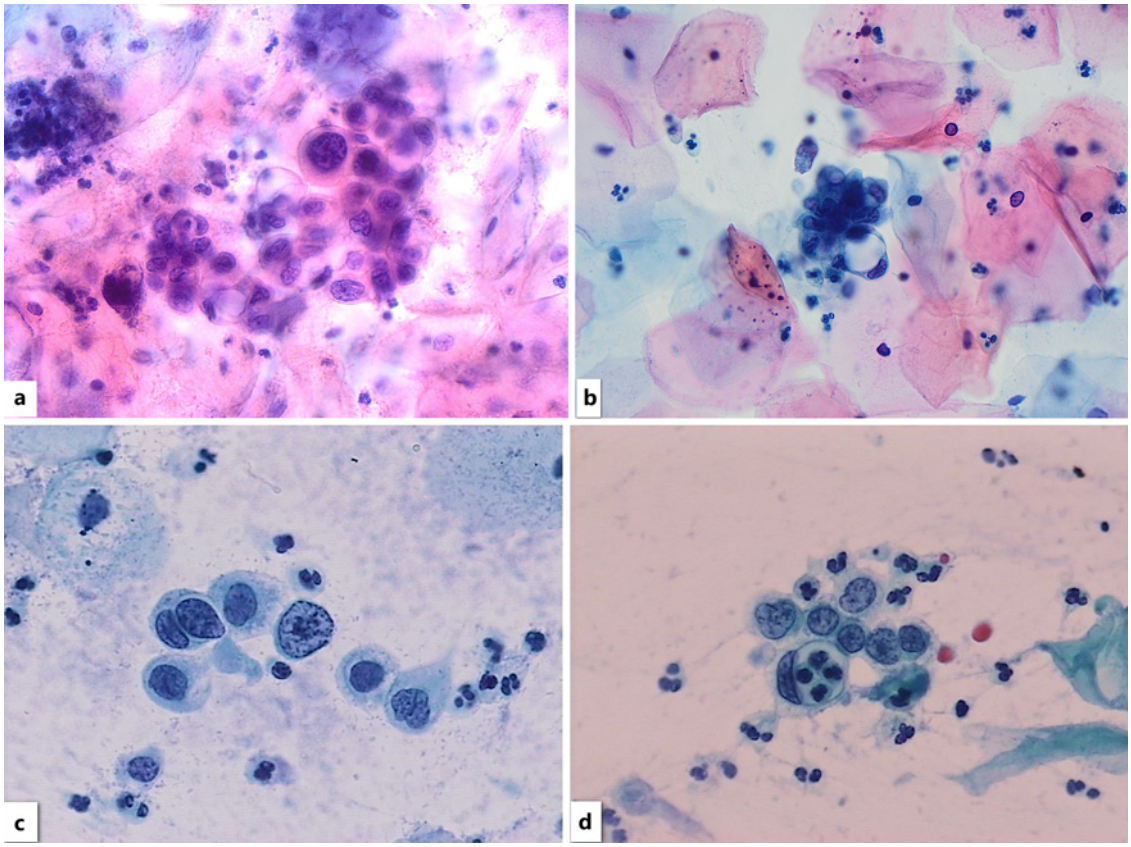

Fig. 5. a Cluster of cells showing IUD change with anisokaryosis and increased N/C ratio. Nuclei are dark, with thick or wrinkled chromatin. Nucleoli and large cytoplasmic vacuolization are observed in a few cells. Papanicolaou, ×400. **b** IUD change presenting as a glandular cluster with large, empty cytoplasmic vacuolization ("signet ring" cell). Papanicolaou, ×400. **c** HSIL, nuclei showing coarse, regular chromatin. Papanicolaou, ×400. **d** Endometrial adenocarcinoma, fine, irregular chromatin, cytoplasmic vacuolization showing phagocytosis of neutrophils. Papanicolaou, ×400. Benign outcome of IUD changes was confirmed by the knowledge of the patient's history and by negative follow-up Pap tests, HSIL, and adenocarcinoma were confirmed by histology.

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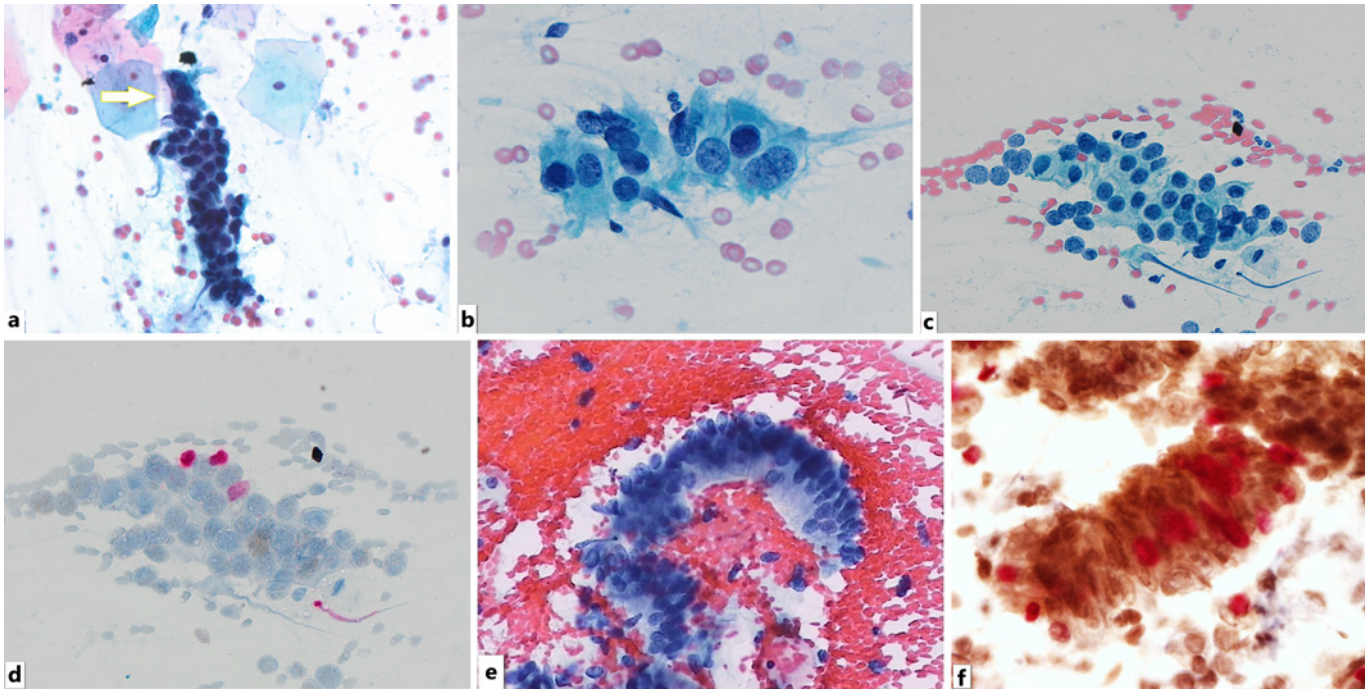

Fig. 6. a Tubal metaplasia showing the stripe of large cells, with increased N/C ratio, hyperchromatic nuclei, and inconspicuous nucleoli. The cells have a ciliated border (arrow). Papanicolaou, ×400. **b** Tubal metaplasia, cilia are barely visible, but terminal plates are observed in a few cells. Papanicolaou, ×600. **c** Tubal metaplasia showing coarse chromatin, without cilia or terminal plates. Papanicolaou, ×400. **d**. the same cluster as in **c**, negative for p16/Ki-67 dual stain. p16/Ki-67 immunostaining, ×400. **e** Stripe of AIS with pseudostratification of elongated nuclei with coarse chromatin. Papanicolaou, ×400. **f** Stripe of AIS positive on p16/Ki-67 dual stain. p16/Ki-67 immunostaining, ×600. Tubal metaplasia and AIS were confirmed by histology.

Nuclei can show degenerative changes, vacuolization, or fragmentation. Mild hyperchromasia can be observed but also hypochromasia and smudgy chromatin. The cytoplasm is pale or amphophilic, sometimes with vacuolization (shown in Fig. 7a–c) [4, 18, 19]. The main differential diagnosis is dysplasia and SCC which have high N/C ratio, dark, irregular nuclei, and irregular, clumped chromatin (shown in Fig. 7d). Keratinized SCC can sometimes resemble radiation change, but in those cases, overt cytoplasmic orangeophilia (keratinization) and tumor diathesis are observed. For correct interpretation, it is important to have information on the radiation therapy provided by the clinician or checked in the patient's electronic chart. After 4 to 6 months since radiation, morphological features of radiation changes gradually subside [18, 19].

Arias-Stella Reaction

Arias-Stella (AS) reaction occurs in association with pregnancy, due to the hormonal impact on glandular endocervical or endometrial cells. These cells are large, can be pleomorphic, with increased N/C ratio, with dark, sometimes irregular nuclei, but the chromatin is usually smooth or smudgy. The cytoplasm is usually vacuolated and pale but can also be dense. They can be present as single cells or in small clusters (shown in Fig. 8). The main differential is HSIL, but it can mimic adenocarcinoma as well [20]. Only smooth, smudgy chromatin characteristics for AS and knowledge of patient history can help to avoid false positive calls. AS reaction can be found in women undergoing clomiphene and beta-human chorionic gonadotropin treatment for infertility [21].

Cells from Seminal Vesicles

Cells from seminal vesicle can occasionally be seen in Pap test. Those cells can resemble HSIL because they can be large, with increased N/C ratio and dark, dense nuclei [22]. These large cells are described in urine cytological samples and showed a broad range of ploidy abnormalities [23]. Usually, they are few and found as single cells or in small clusters or rows. When these atypical cells

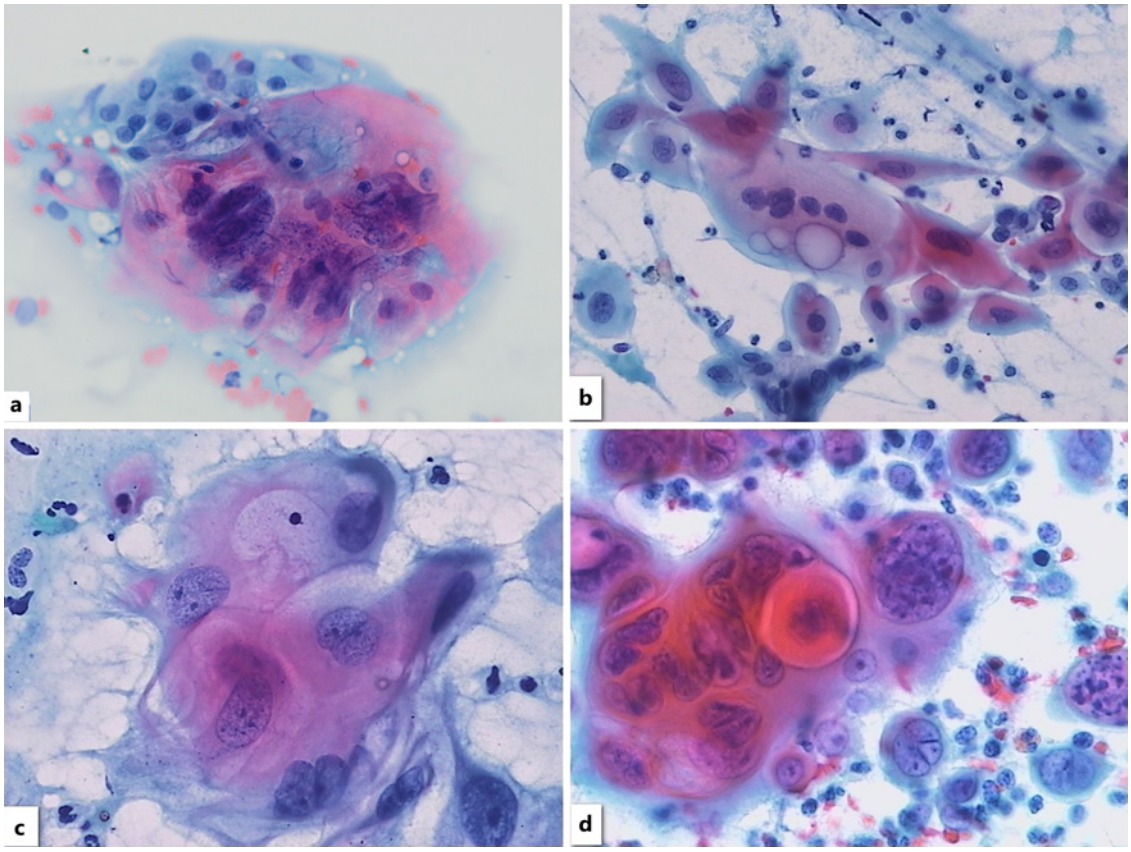

Fig. 7. a Radiation changes of the squamous epithelium showing enormously large cells with multinucleation but without increased N/C ratio. Papanicolaou, ×400. **b** Radiation change with bizarre cellular shapes, smooth chromatin, no prominent nucleoli, and low N/C ratio. Vacuolization and amphophilia of the cytoplasm are observed. Papanicolaou, ×400. **c** Radiation change with nuclear and cytoplasmic degenerative changes. Papanicolaou, ×400. **d** SCC with pleomorphic malignant cells showing dark, irregular nuclei, clumped chromatin, and irregular nucleoli. Keratinization is observed as orangeophilia of the cytoplasm. Papanicolaou, ×400. Radiation changes were diagnosed with the knowledge of the patient history, and SCC was confirmed by histology.

are observed in the background of spermatozoa, the origin of seminal vesicles could be suspected (shown in Fig. 9). In the follow-up, it could be recommended to repeat the smear after 3 to 4 days of abstinence.

Pemphigus Vulgaris

Pemphigus vulgaris can affect skin and mucosa, including a rare involvement of the cervicovaginal area. In cervical smear, it can show high cellularity of dispersed, acantholytic squamous cells of parabasal or deep intermediate type due to the loss of intercellular connections, with round to oval nuclei and conspicuous nucleoli. In some cases, round or glandular-like clusters can be found (shown in Fig. 10). This pattern can be mistaken for cancer, either squamous or adenocarcinoma, especially if the cytopathologist is not aware of the patient's condition. The main differences are the relative uniformity of the cells in the pemphigus, lack of severe nuclear atypia, and absence of tumor diathesis. The patients usually present with vaginal discharge, dyspareunia, and vaginal bleeding, but can be asymptomatic [24].

Hyperchromatic Crowded Groups

HCG is a well-described morphological feature in Pap test found in varieties of entities. It can be a source of false negative and false positive results. It usually represents benign tissues such as squamous metaplasia, atrophy, reactive glandular cells, tubal metaplasia, and endometrium originating from the lower uterine segment (LUS), but the differential diagnosis includes HSIL and AIS [4, 25–27]. Morphological characteristics of HCG are shown in Table 4.

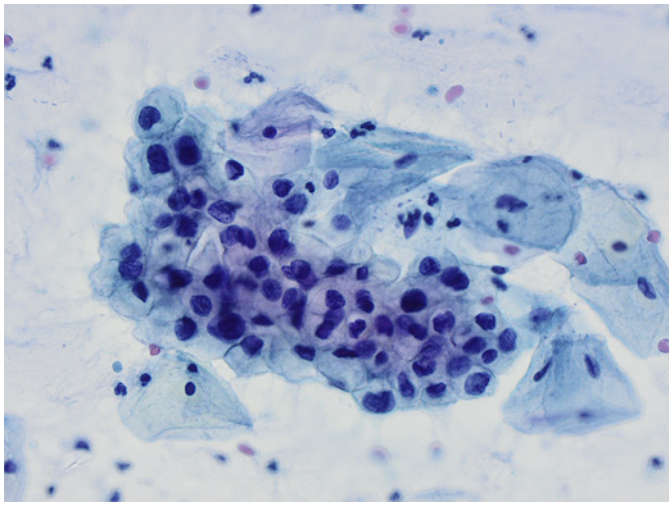

Fig. 8. The cluster of cells with Arias-Stella changes which show dark irregular nuclei with indentations, smudgy chromatin, and increased N/C ratio. The cytoplasm is pale resembling the cytoplasm of endocervical glandular cells. Papanicolaou, ×400 Diagnosis was supported by the knowledge of the patient's current pregnancy.

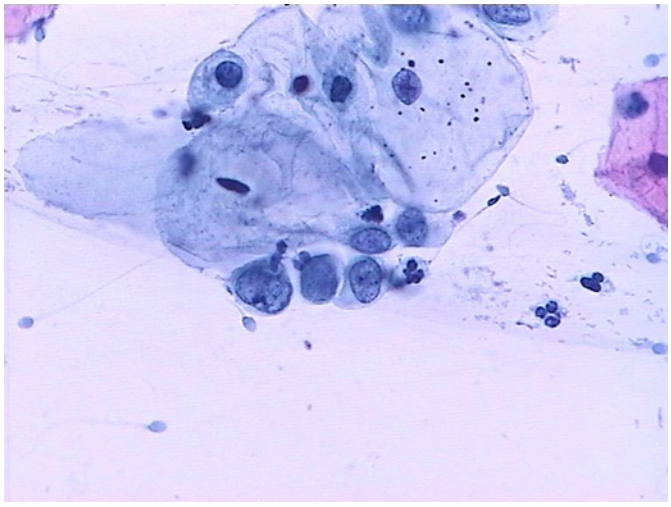

Fig. 9. Cells from seminal vesicle which show increased N/C ratio and irregular nuclear border. Spermatozoa are found in the background. Papanicolaou, ×400. Diagnosis was supported by the follow-up negative Pap tests.

HCG representing HSIL can be recognized by syncytial clusters with loss of nuclear polarity. The nuclei are often monomorphic. Sometimes, in the cluster, one or few nuclei can be two or three times larger compared to the others, small- or medium-sized nuclei. Nucleoli are inconspicuous, but occasional mitoses can be seen. Nuclear features of HSIL

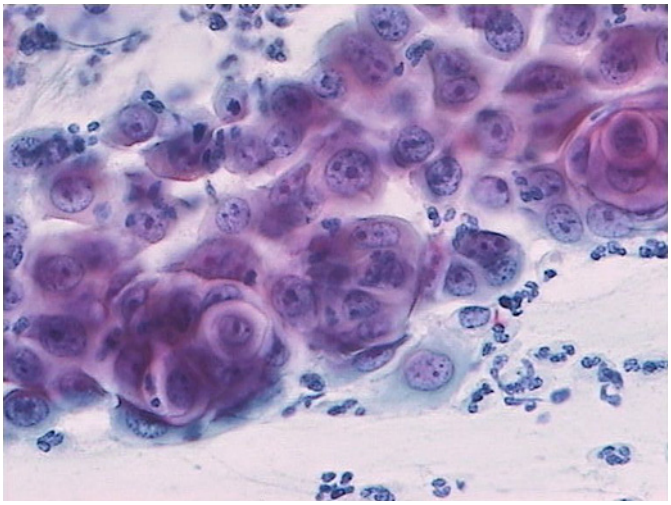

Fig. 10. Cervical smear of the patient with pemphigus vulgaris showing acantholytic squamous cells of parabasal and deep intermediate type, with round to oval nuclei and prominent nucleoli. Few glandular-like and cell-in-cell clusters are observed. Papanicolaou, ×400. Diagnosis of the pemphigus vulgaris was confirmed by histology.

can be better observed at the margins of the cluster (shown in Fig. 11a, b). In AIS, the clusters show typical nuclear elongation and feathering, and regular coarse chromatin (shown in Fig. 11c). In benign glandular or immature squamous metaplastic cells presenting as HCG, nuclei are uniform, round to oval, with smooth chromatin and regular nuclear outline (shown in Fig. 11c). Nuclear polarity is preserved. In squamous epithelium, cytoplasmic borders are preserved, and in glandular epithelium smooth border of the cluster can be found at least partially. When the endocervical smear is taken vigorously with the brush, endometrial epithelial and stromal cells from the LUS can appear in the cervical smear and present as HCG. In those cases, tubular glands with attached stroma are observed. Endometrial cells are dark and small, nuclear borders can be irregular due to the shrinkage effect (shown in Fig. 11e, f). Interpretation of HCGs is especially challenging in LBC, compared to CSs. In LBC, the cell clusters are usually very dark and three-dimensional, but the same morphological criteria are applied as described above [26]. Dual immunocytochemistry staining for the biomarker p16/Ki-67, which is usually positive in HSIL, could be helpful in the differential diagnosis [28].

Biomarker p16/Ki-67

Biomarker p16/Ki-67 represents a simultaneous expression of p16 protein, which is a surrogate marker for HPV oncogenic infection, and proliferation marker, Ki-67. The method used for the p16/Ki-67 application is a dual

Table 4. HCGs – morphological characteristics and p16/Ki-67 immunocytochemistry

	Squamous metaplasia	Atrophy	Reactive glandular cells	Tubal metaplasia	LUS endometrium	HSIL	AIS
Nuclear size	Small, medium	Small, medium	Medium	Medium	Small	Small, medium, occasional few large nuclei	Medium, elongated
Nuclear size variation	Absent	Usually absent	Absent	Absent	Absent	Absent	Absent
N/C ratio	Moderate to high	Moderate to high	Moderate	Moderate	High	High	High
Nuclear polarity	Preserved	Preserved	Preserved	Preserved	Preserved	Disturbed	Variable
Chromatin	Smooth, dense	Fine, smooth, dense, or smudged	Fine	Dark, regular	Fine, sometimes dark	Coarsely granular, regular	Coarsely granular, regular
Nuclear chromasia	Slightly hyperchromatic	Hyperchromatic	Slightly hyperchromatic	Hyperchromatic	Hyperchromatic	Hyperchromatic	Hyperchromatic
Nuclear border	Regular	Regular	Regular	Slightly irregular	Regular	Irregular	Regular or slightly irregular
Nucleoli	Absent	Absent	Small nucleoli can be present	Can be present	Absent	Absent	Absent
Mitoses	Absent	Absent	Absent	Absent	Absent	Present	Present
Cytoplasmic features	Basophilic, dense	Basophilic, fine, poorly defined	Basophilic or amphophilic, vacuolised	Basophilic, columnar, with ciliated borders or terminal plates	Basophilic, scant, poorly defined	Basophilic, scant	Basophilic, columnar
Cytoplasmic borders in clusters	Present	Absent	Present, outer borders smooth	Variable	Absent	Absent	Variable
Type of clusters	Solid, sheets, three-dimensional	Solid, sheets, three-dimensional	Three-dimensional, glandular features	Palisades, sheets	Sheets or three-dimensional, tubular, attached stroma	Solid, syncytial, three-dimensional	Palisades, stripes, rosettes, feathering
p16/Ki-67	Negative	Negative	Negative	Negative	Negative	Positive	Usually positive, can be negative in HPV-independent types

LUS, lower uterine segment; HSIL, high-grade intraepithelial lesion; AIS, adenocarcinoma in situ; N/C ratio, nucleo-cytoplasmic ratio.

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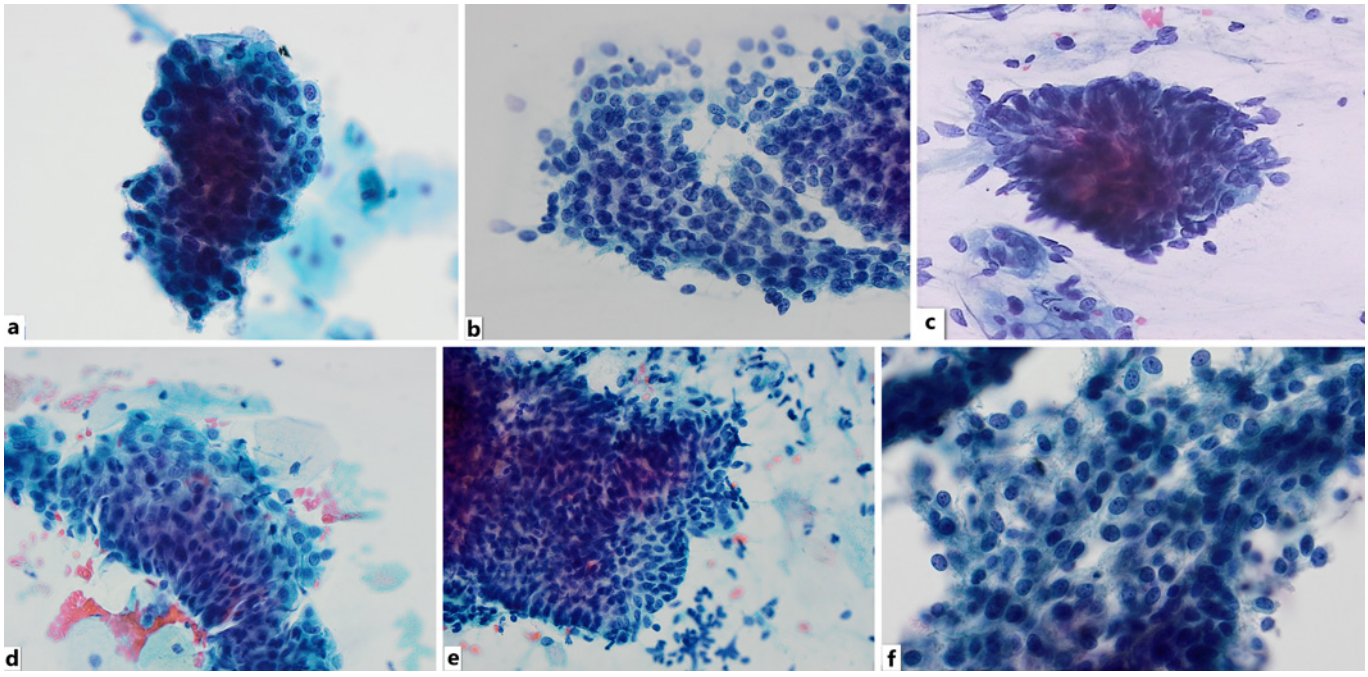

Fig. 11. a HSIL presents as HCG with a syncytial cluster with loss of nuclear polarity. The nuclei are monomorphic with irregular nuclear membranes. In liquid-based preparation, nuclear features of HSIL are better observed at the margins of the cluster. Papanicolaou, ×400. **b** HSIL with monomorphic nuclei and coarsely granular chromatin. Papanicolaou, ×400. **c** AIS with elongated, overlapping nuclei, and nuclear feathering at the margins. Papanicolaou, ×400. **d** HCG of immature benign metaplasia with oval nuclei and smooth chromatin, cellular borders are preserved. Papanicolaou, ×400. **e** HCG of benign endometrial epithelial cells with small oval nuclei, smooth chromatin, and nuclear polarity preserved. Papanicolaou, ×400. **f** endometrial stromal cells, dispersed, with small oval, normochromatic nuclei and fine chromatin. Papanicolaou, ×600. HSIL and AIS were confirmed by histology, benign HCG cases had negative follow-up Pap tests.

immunocytochemistry staining on cytological preparations of Pap test, both on CSs and LBC. A positive reaction is the finding of at least one positive cell with double reaction, brown nucleus, cytoplasm (positive p16), and red nucleus (positive Ki-67). Increased specificity is observed when cut-off number of positive cells is three, compared to 1 cell [29]. Biomarker p16/Ki-67 has sensitivity comparable with HPV test but shows better specificity in detecting HSIL. It is mostly used in triaging borderline and low-grade cytology for referral to colposcopy [30]. It is useful in detecting glandular dysplasia and cervical adenocarcinoma [31]. In the study of clinical validation of p16/Ki-67 dual stain, it showed better risk stratification than cytology and provided high reassurance against pre-cancers, irrespective of the HPV genotype [32].

Tumor Diathesis

Tumor diathesis (TD) in the cases of advanced invasive cancer can be the sole feature observed in cytology without any preserved and recognizable malignant cells, and results in false negative reports. TD is composed of a mixture of fresh and disintegrated erythrocytes and leucocytes, cellular debris, and proteinaceous material (shown in Fig. 12a, c). Sometimes, only a few malignant cells or malignant naked nuclei can be found (shown in Fig. 12b, d). In CSs, TD gives an impression of the red-grayish substance covering the hole slide, and in LBC it is present as a patchy or granular material sticking to the cells. In LBC TD can block the filter resulting in decreased cellularity [33]. When observed, the first action is to rescreen the slide more carefully, and if no suspicious or malignant cells are found, accentuate this finding in the report recommending further investigation. The importance of tumor diathesis, even if it is included in the negative results of the Pap test, should be understood by clinicians, and always raise suspicion of malignant disease. Background like tumor diathesis can occur in severe inflammation, atrophy, and smears with nabothian cyst contents which can also contain proteinaceous material, neutrophils, or debris [5, 34].

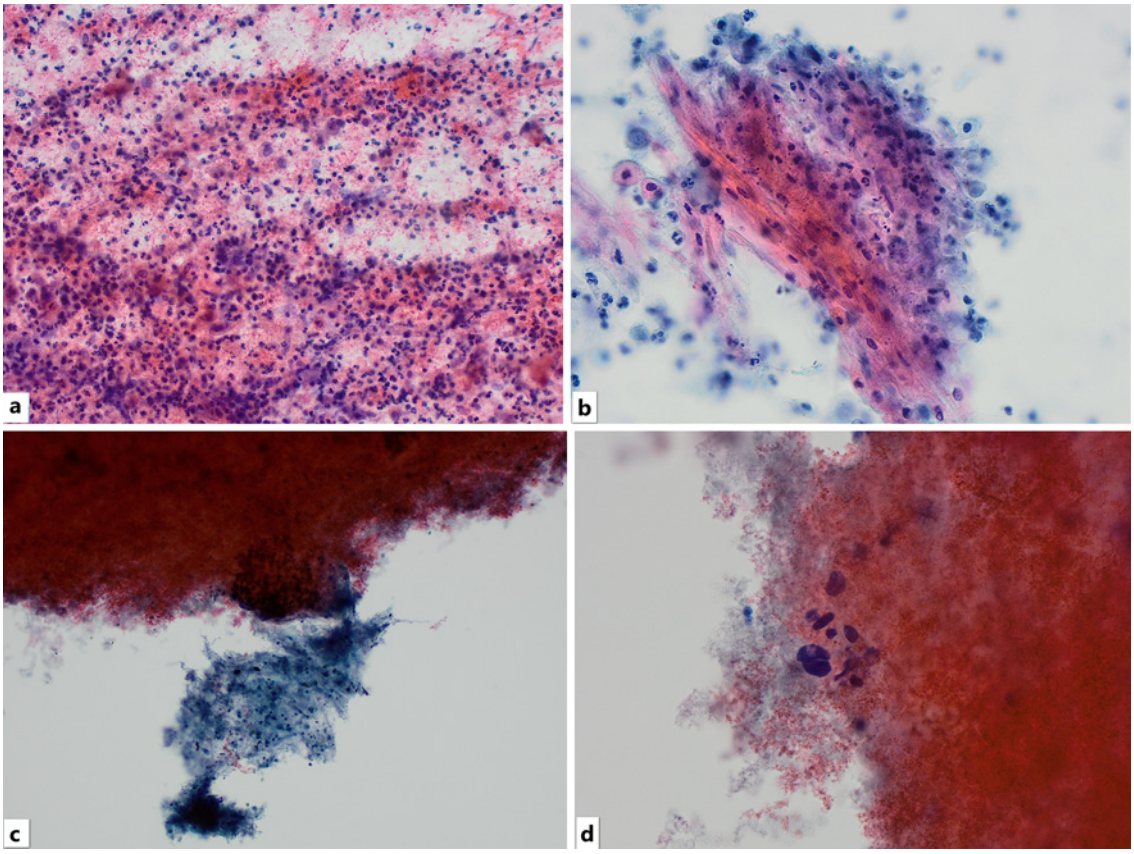

Fig. 12. a Tumor diathesis composed of a mixture of fresh and disintegrated erythrocytes and leucocytes, cellular debris, and proteinaceous material without any malignant cells. Papanicolaou, ×200. **b** Tumor diathesis with few degenerated spindle malignant cells of the keratinized SCC. Papanicolaou, ×400. **c** LBC preparation showing dense granular, bloody debris of the tumor diathesis. Papanicolaou, ×400. **d** Only a few malignant naked nuclei in the background of granular and bloody debris in the LBC sample. Papanicolaou, ×400. SCC was confirmed by histology.

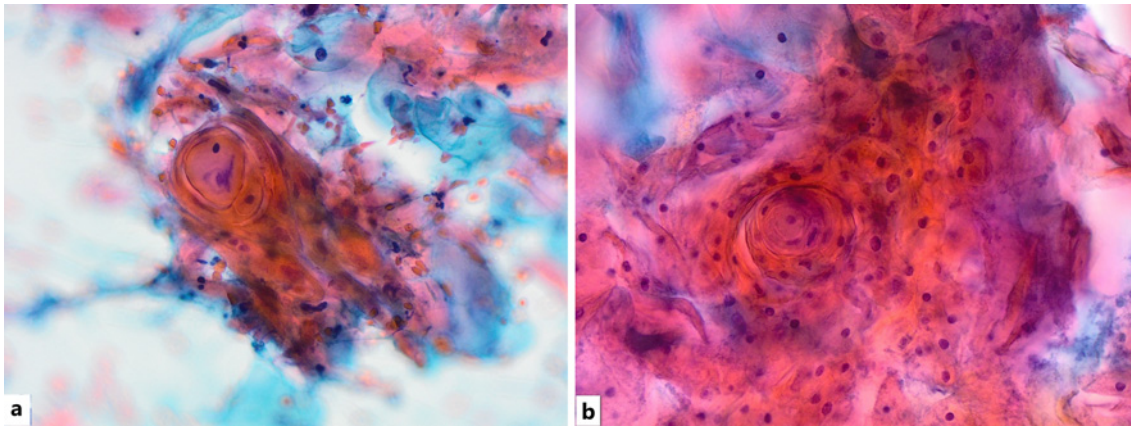

Fig. 13. a Keratinizing SCC showing atypical keratotic cells with low N/C ratio. Nuclei are ink-dark punctuated or elongated, but small. Cytoplasm is dense, orangeophilic, or yellowish. Papanicolaou, ×400. **b** Benign keratin pearl with regular shape. Nuclei are pycnotic or with fine chromatin. Papanicolaou, ×400. SCC was confirmed by histology, and the case showing benign keratin pearl had negative cytological follow-up.

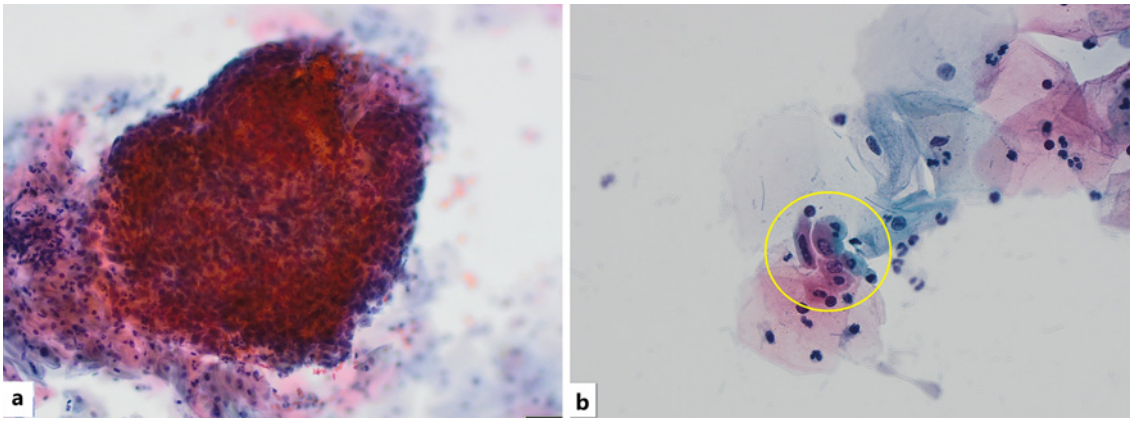

Fig. 14. a Papillary SCC. Large, dense fragment of keratotic cells with slight atypia, Papanicolaou stain, ×200. **b** Group of extra small ASCs with dark coarse chromatin (circle), Papanicolaou stain, ×400. Papillary SCC was confirmed by histology.

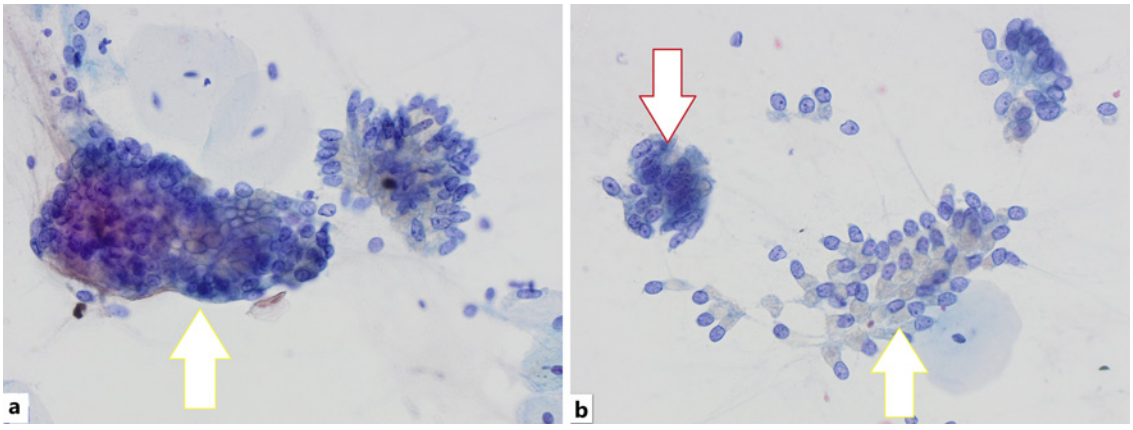

Fig. 15. a Gastric type adenocarcinoma. "Drunken," irregular honeycomb arrangement of atypical glandular cells (arrow), Papanicolaou stain, ×400. **b** Atypical glandular cells with conspicuous nucleoli (red arrow) and yellow mucinous cytoplasm (yellow arrow), Papanicolaou stain, ×400. Gastric-type adenocarcinoma was confirmed by histology.

Keratinizing Squamous Cell Cancer

Well-differentiated SCC with keratinization, including verrucous carcinoma, is covered with keratinized cells on the growing surface, which can present in cytology as atypical parakeratosis, without classical malignant features. So, it can be a source of false negative or underdiagnosis in cytology usually reported as keratotic ASCs of undetermined significance (ASCUS). Morphological characteristics which can help in giving a correct diagnosis in cytology are hypercellular smear with abundant keratotic cells with low N/C ratio, spindle, or tadpole cells. The cytoplasm is dense, orangeophilic, or yellowish. The cells can form irregular keratin pearls. Nuclei are ink dark, often elongated or needle shaped (shown in Fig. 13a). Tumor diathesis is usually present [4, 35]. On the other hand, benign keratin pearls show a more regular shape, and nuclei are pycnotic or with smooth chromatin (shown in Fig. 13 b).

Papillary Squamous Cell Cancer

Papillary squamous cell carcinoma of the uterine cervix is a rare, distinct morphological variant of SCC [36]. These tumors are HPV-associated. Thin or broad papillae with connective tissue stroma are covered by epithelium with features of LSIL or HSIL, so a superficial biopsy may not reveal evidence of invasion. Complete excision of the

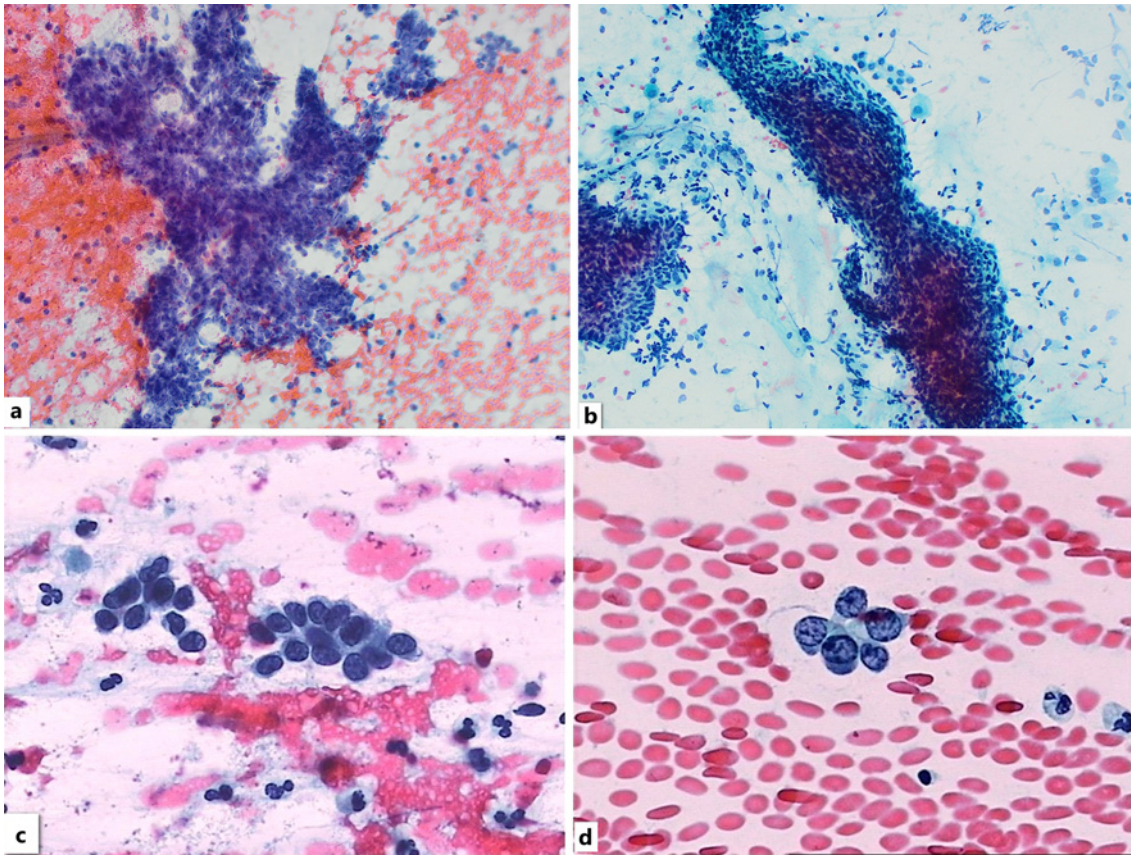

Fig. 16. **a** Well-differentiated endometrioid adenocarcinoma presenting as an irregular and branching cluster with pseudoluminal spaces. Nuclei are round to oval with irregular chromatin. Papanicolaou stain, ×200. **b** Cluster of the LUS with epithelial and stromal cells, polarity is preserved, nuclei are small and oval with smooth chromatin. Papanicolaou stain, ×200. **c** "Normal" appearing hyperchromatic endometrial cells. Papanicolaou stain, ×400. **d** Atypical endometrial glandular cells with mild anisonucleosis, irregular chromatin in small rosette formation. Papanicolaou stain, ×400. For the cases shown in figures **a** and **d**, endometrioid adenocarcinoma was confirmed by histology, benign nature of the cases in figures **b** and **c** was confirmed by clinical follow-up.

clinically visible lesion usually reveals an underlying invasive tumor [37]. According to the histological pattern of this tumor, it is not surprising that cytological smear often shows only features of ASCUS or LSIL with keratotic changes. In the study of LBC morphology of papillary SCC, mostly basaloid squamous cells were observed but some with bizarre or fiber shapes, with clearly atypical nuclei [38]. The most prominent features in cytology are large, hypercellular clusters of parakeratotic cells with slight cellular atypia, extra small ASCs with high nucleo-cytoplasmic (N/C) ratio, nuclei with irregular borders, dyskeratotic small cells, and weakly stained, pale, atypical squamous "ghost" cells (shown in Fig. 14a, b). HCG, if found, represents a deeper layer of the cancerous tissue, and can help with the right diagnosis in cytology. These cancers in colposcopy have characteristic "warty" appearance and should raise suspicion when corresponding cytology and histology are of the low grade, resulting in repeat, deeper biopsy, or cone biopsy [39].

Gastric Type Adenocarcinoma

Most of the adenocarcinomas of the uterine cervix are correctly recognized and diagnosed on a Pap test. Factors leading to under-calling are low cellularity or low-grade morphology of glandular tumor cells [40, 41]. Gastric type adenocarcinoma of the uterine cervix is a well-described entity. It is an HPV-independent cancer and accounts for about 10–15% of cervical adenocarcinoma cases. In histology, the tumor is composed of glands lined with cells with abundant, pale cytoplasm and distinct borders. Morphology ranges from extremely well-differentiated adenocarcinomas with deep stromal gland distribution to poorly differentiated cancers [37, 42]. If well differentiated, it can be a source of false negative findings or underdiagnosis on cytology and histology. Cells and glands show minimal morphological abnormalities, which can cause uncertainties in interpretation. Pap tests are usually reported as atypical endocervical cells

(AGC) of endocervical origin because of minor morphological changes, without displaying typical morphological characteristics of the usual type of cervical AIS or adenocarcinoma. The cells and groups originating from GTA are usually monomorphic, arranged in irregular, "drunken" honeycombs, rosettes, or rigid stripes. Nuclei are round to oval, with smooth chromatin, focally with conspicuous nucleoli. The cytoplasm is typically tall and filled with yellow-stained mucin on Papanicolaou staining, a distinctive feature which can raise a suspicion of gastric differentiation [43] (shown in Fig. 15a, b). These cancers are HPV independent, so HPV testing can also be misleading. Described morphological features are much more prominent in the conventional cytological smear compared with LBC preparation [44].

Endometrioid Adenocarcinoma

Endometrioid adenocarcinoma usually originates from endometrium but can also represent a rare type of endocervical cancer. It is HPV independent [37]. If well differentiated, in cervical smear, it can be interpreted as normal endometrial cells from the LUS giving rise to a false negative result. Well-differentiated endometrioid adenocarcinoma forms irregular and branching clusters with pseudoluminal spaces with round to oval nuclei and irregular chromatin (shown in Fig. 16a). Clusters of the LUS usually present as a combination of epithelial and stromal cells, with preserved polarity and small oval nuclei with smooth chromatin (shown in Fig. 16b). In postmenopausal women, a finding of "normal" appearing endometrial cells in Pap test is an abnormal finding which warrants further investigation (shown in Fig. 16c) [4, 45]. In premenopausal or perimenopausal women, endometrioid adenocarcinoma, if misinterpreted by cytology as a normal finding, can lead to a delay in diagnosis. In the cytology of such cases, observation of the slightest abnormalities in endometrial cells must result in at least AGC diagnosis. These are mild anisonucleosis, chromatin irregularities, small prominent nucleoli, clusters or fragments with irregular glands, loss of polarity, rosettes, or papillary group formations (shown in Fig. 16d). Tumor diathesis can be found focally or diffusely. On the other hand, benign endometrial cells of the LUS can be misinterpreted in Pap test as glandular atypia or adenocarcinoma, as well as a rare condition of cervical endometriosis [46, 47].

Conclusion

Pitfalls in Pap test presented in this paper are the selection of examples and entities, but many more sources of errors can be encountered in Pap test interpretation. They include unsatisfactory samples, the presence of lubricants or artifacts, non-HPV viral infections, microglandular hyperplasia, metastatic tumors, non-epithelial neoplasms, lymphocytic cervicitis, samples from transgender patients, endometrial cells in menstruation, decidual changes, vitamin deficiencies, and other challenging categories.

The awareness of pitfalls in cervical cytology, and the knowledge of mimics and differential diagnosis is crucial for cytotechnologists and cytopathologists to minimize errors. However, on the institutional level, cytology laboratory quality assurance (QA) protocols must be implemented, following national or international recommendations. These include professional and technical guidelines for assuring the collection and preparation of an adequate cervical cell sample, adequate handling and staining of the samples, screening and interpretation of the slides, and uniform reporting of the results [48, 49]. Rescreening of the negative slides is another important measure for QA, as well as monitoring Pap test reporting rates, implementation of dashboards for performance metrics, and cytologic-histologic correlation guidelines [50–52]. Review of Pap tests with erroneous diagnosis is mandatory, and it must be considered an educational- and experience-building procedure allowing cytologists to learn new features and keys to correct diagnosis. Also, clinicians must be informed about the entities which can lead to false negative or false positive outcomes of the Pap test. Multidisciplinary team meetings comprising gynecologists, radiologists, oncologists, pathologists, and cytopathologists, during which all discrepant cases and results will be discussed, are important for better patient care. On the other hand, cytopathologists should not pull back in significant diagnoses, especially in HPV-negative cases.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

D.V.-M.: original draft, methodology, and writing. S.S.-P., D.V.O., and R.R.: investigation, references, and selecting cytology slides. M.K.: investigation and evaluation of clinical considerations. S.E.: evaluation of histology and writing.

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