Non-gynaecological cytology I

Methods and preparatory techniques in non-gynaecological cytology (special stains, cell blocks, ICC, FISH and CISH)

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Introduction

Diagnostic cytological criteria are based on which sample preparation is used. Methods for preparation, fixation and staining vary from laboratory to laboratory, but the basic principles are the same. The goal is to reduce cellular artefacts and obtain an optimal diagnostic specimen.

Fixation

The purpose of fixation is to preserve cell morphology, replace cellular water, facilitate diffusion of staining molecules, deactivate autolytical enzymes and prevent growth of microorganisms. Cells may be fixed in alcohol wet fixed (methanol or ethanol) or sprayfixed (ethanol and Carbowax). If immediate preparation is available, a fresh specimen without preservative is the best.

Lysing red blood cells

Different solutions may be used to lyse red blood cells in heavily bloody smears, e.g. Carnoy´s fixative (always prepare fresh): absolute ethanol, chloroform, glacial acetic acid (6:3:1) and Clarke´s solution: absolute ethanol, glacial acetic acid (3:1). Whatever method used, always rinse the slide thoroughly in 95 % ethanol, after lysing solution and before staining, in order to stop the reaction of the lysing solution. CytoLyt/10 % acetic acid can be used to lyse red blood cells in bloody specimens for liquid-based preparations.

Cytocentrifuge preparation

This involves a special centrifuge that spins small specimens of fluid directly on to a slide. The amount of fluid placed in the cytocentrifugation

chamber, depends on the concentration of cells in the fluid. The samples may need to be diluted or concentrated before use. The preparations create small cell deposit in a monolayer pattern and may also be used for immunocytochemistry.
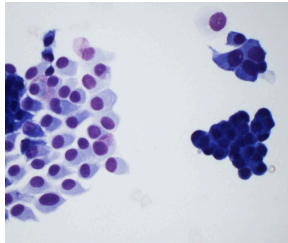
The Papanicoloau staining method

The Papanicoloau staining method is used to stain alcohol fixed specimens. The method uses the dye haematoxylin to stain the nuclei and two cytoplasmic stains (OG and EA). The staining method brings out distinct nuclear details and cytoplasmic keratinization. (See more details in tutorial notes: Gynaecological cytology: technical aspects.)

Romanowsky staining method

The Romanowsky staining method is mainly used to stain air-dried specimens, which are fixed in methanol. The method uses three dyes: methylene blue, azure B and eosin Y. May-Grünvald-Giemsa is one example of a Romanovsky staining. The staining result should achieve purple nuclei, blue cytoplasm and pink, red blood cells. The method is superior for visualization of mucus, colloid, stroma and cytoplasmic details like granules and inclusions.

Rapid staining techniques

Rapid staining techniques are used at the bedside of the patient, to ensure that adequate material has been obtained and sometimes a preliminary diagnostic assessment can be made. Several rapid stains are available, e.g. Rapid Papanicolaou stain and Diff-Quik stain (a modified Romanowski staining) with a reduced staining time to approx. 2 minutes.

Cell blocks

Cell block technique or paraffin embedding of sediments of fluid is useful both for morphological evaluation due to the recognition of histological

patterns of disease and especially immunocytochemistry. FNA, serous effusions, urine sediment and material from gastrointestinal tract are suitable for cell block preparation. Residual material from ThinPrep preparation may also be processed as cell block. Example of two methods for cell block preparation are the Agar method and plasma-thrombin clot method:

Centrifuge the cell suspension for 10 minutes for appropriate rpm.

1. **The Agar method**: The sediment is placed on a paper towel and transferred to a small pool of melted 3% agar. Ensure that the whole sediment is covered in agar. Let stand for a few minutes to harden. Avoid creating air bubbles. Trim the excess agar and place the agar button in a tissue cassette.

2. **Plasma-thrombin clot method**: Sediment from fresh unfixed specimen are mixed with a few drops of outdated blood plasma from the blood bank. Plasma may be coloured with a small amount of a colour such as eosin. Add one drop of thrombin solution and five drops of plasma. This mixture will clot in 1 to 2 minutes if the reagents are fresh and not cold. The clot is wrapped in lens paper and placed in a tissue cassette. If the specimen was prefixed with alcohol, the sediment must be washed with balanced salt solution, since alcohol inhibits the clotting action of plasma and thrombin.

Place the tissue cassette in the fixative recommended for cell block. Process as for tissue.
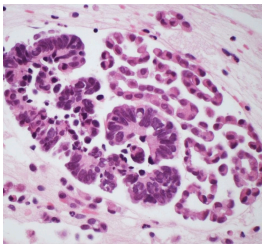
Immunocytochemistry (ICC)

ICC identifies properties of cells and tissue through antigen (Ag)-antibody (Ab) interactions. The utility of ICC in diagnostic cytopathology consists of supplemental information in cancer diagnostics, evaluation of predictive markers (ER, PR, HER 2) and prognostic information (Ki-67). Different preparations (smear, LBC, cytospin and cell block) can be used for ICC. Prerequisite for adequate ICC are well-spread film of cells on a glass slide, adequate fixation, removal of blood and proteinaceous material and a sensitive, reproducible method of ICC. It is well known that

immunocytochemistry on cytological specimens is far from being standardized.

Fixation immobilise antigens, preserve the antibody binding activity, cell and tissue architecture and permeability for immune reagents. Wet fixation into alcohol must be performed without delay, because air-drying may result in false positive results. Air-dried smears must be fixed immediately before ICC processing. The fixative of choice, vary among cytopathologists/cytotechnologists. Cold acetone and 95 % alcohol are common fixatives, but remember, some antibodies do not work well with primary alcohol fixatives. The type of fixative depends on which antigen is examined.

Examples of ICC staining principles

- **ABC method**: An unlabelled primary antibody (Ab) reacts with cell antigen (Ag). A labelled secondary Ab reacts with the primary Ab. The third layer is a complex of avidin-biotin peroxidase. When adding DAB or other chromogen substrate, the complex produces a coloured product, visible in a light microscope.

- **Polymeric method**: Polymer-conjugated systems use a polymer spine (dextran) with multiple enzymes and antibodies as the second layer, which reduces the number of assay steps. Another advantage of polymer-conjugated systems, are that the dextran backbone has no affinity to tissue protein and the result is little endogenous binding. Avidin-biotin methods on the other hand, bind to endogenous biotin in tissues.

Internal quality control

A positive control (PC) must be positive and fixed in the same way as the diagnostic samples to show the proper performance of the staining reagents and methods.

A negative control (NC) is added to assess non-specific ICC staining due to non-specific background staining or lack of Ab specificity. If positive staining occurs in NC, the test specimen result is invalid.

External quality control

Participating in ICC quality control schemes such as UK NEQAS is recommended. A review of UK NEQAS ICC (cytology module) showed that a variety of cytology sample types (cytospins, smears, LBC and cell

block) are in use and fixed with different fixation protocols used for immunocytochemistry (reference below). The quality of immunostaining was on average good, regardless of the slide preparation and fixation technique. Cell block preparations though, achieved significantly better scores than the other. As cytology preparations are different than cell block, they require optimal adjustment with respect to primary antibody dilution and pre-treatment procedures.

Fluorescence and chromogenic in situ hybridization (FISH and CISH)

FISH visualises specific DNA sequences, by using fluorescent-labelled probes and can be used for quantification of chromosomes and genes, e.g. HER2/neu expression in breast cancers, to identify those who may benefit from Trastuzumab (Herceptin) treatment. After hybridization (binding) to the complementary DNA on the slide, the probes can be visualised with a fluorescence microscope. The hybridization succeeds in > 95 % of routine cytologic specimens, when it is done within a few weeks after preparation. Another method for detecting HER2/neu is chromogenic in-situ hybridization (CISH). CISH uses an immunoperoxidase reaction to visualize the HER2 probe, which requires only a conventional light microscope. Furthermore, slides can be kept permanently due to staining remains stable.

References

- Comprehensive Cytopathology, ed. Bibbo M, Wilbur D, 3th ed., Saunders Elsevier, 2008.

- Koss´ Diagnostic Cytology and its Histopathological Bases, ed. Koss LG, Melamed MR, 5th ed., Lippincot Williams & Wilkins, 2006.

- Diagnostic Cytopathology Essentials, ed. Kocjan G, Gray W, Levine T, Kardum-Skelin I, Viehl P. Elsevier 2013.

- Cytopreparation, Principles and Practice (Essentials in Cytopathology), Gill G, Springer 2013.

- External quality control for immunocytochemistry on cytology samples: a review of UK NEQAS ICC (cytology module) results, I.S. Kirbis et al. Cytopathology 2011; 22: 230-7.

- Immunocytochemistry: an indispensable technique in routine cytology, L. Skoog and E. Tani. Cytopathology 2011; 22: 215-29.

- Current technologies for HER2 testing in breast cancer. Moelans CB et al., Critical Reviews in Oncology/Hematology 2011; 80: 380-92.

URINE CYTOLOGY

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Practical information for workshop cases

In each workshop the cases include anagraphical and clinical data about patients and the time of diagnosis on the front of the package. There is usually one slide per package, Papanicolaou stain, but in some packages there are three slides taken on different days.

Suggestion is to examine the slides first, get an idea and then look on the back of the package. There you will find the cytological diagnosis according to our classification, the main points in the cytological description of the slides, the histological diagnosis for those patients who have been operated on and the data about the patients who have been following up by clinicians. Most samples are voided urine and there are some samples of catheterised and ileal conduit urine and the washing from ureter or bladder.

Introduction

WHY Urine Cytology

Aim of Urine Cytology (UC) is the detection of Urothelial Cell Carcinoma (UCC) and related conditions of the pelvis, urethers, and bladder. This is done by cytology of voided urine and/or by lavage during cystoscopy.

In the last decade, many urine-based tests for UCC have been developed and tested with unsatisfactory results, but for urologists, considering the amount of follow-up cystoscopies, UC remains the first choice for a series of reasons, although urine markers for recurrent disease would be useful.

UC of voided urine is the unique non-invasive method of examination of UCC, with high Validity in detection of **high-grade UCC**, but with low

performance in cases of **low-grade UCC**, even with the help of ancillary techniques.

Different benign conditions and diseases such as viral infections and types of haematuria as well as for recognition of the effects of various therapeutic procedures (e.g. organ transplantation) can be effectively detected and diagnosed by UC.

WHEN UC

UC is obviously decided by the urologist or the family doctor who has in charge the patients, based on his work settings and presentation symptoms, namely screening in subjects exposed to carcinogens, triage of symptomatic patients, detection of non-neoplastic, premalignant and malignant urinary lesions, metastatic tumors and lesions inaccessible to cystoscopy or biopsy, follow-up and monitoring the therapy.

WHO

Microscopy is performed by a cytotechnologist (screening) and a pathologist (rescreening).

Cytotechnologists perform the first **analysis:**

1) Qualitative assessment of ADEQUACY
2) PRESENCE of erythrocytes (in voided urine only) distinguishing smooth erythrocytes with intact membrane and dysmorphic ones with wrinkled membrane
3) Qualitative analysis of glomerular erythrocytes and casts (hyaline and granular in normal urine and erythrocytic, leukocytic, epithelial, waxy, mixed cell casts in pathological urine).

The qualitative analysis includes EVALUATION of

Background (clean, slightly dirty and dirty),

Cellularity (rare, few, moderate or many cells)

Detection of cells and non-cellular elements like casts, crystals (uric acid, calcium oxalate and triple phosphatase in normal urine and pathological

crystals like cystine, tyrosine, leucine, bilirubin, drugs) and contaminants (powder, cotton, fibrin, lubricant, mucus, corpora amylacea)

Epithelial cells are urothelial, squamous, columnar and renal epithelial cells.

Non-epithelial cells are neutrophils, erythrocytes, eosinophils, histiocytes and macrophages

The semiquantitative analysis includes the differential cell count of glomerular and non-glomerular erythrocytes in cytospin sediments of voided urine only.

Urine Collection

The **types of urine** for UC are

- early morning voided urine (the most common),
- catheterised and
- organ conduit urine, most often an ileal one

Sampling and processing

The optimal specimen for UC is the second-in-the-morning, freshly voided urine obtained by the clean-catch technique taken for 3 consecutive days

Recommendations for patients:

Acidification of Urine: drink a lemonade or better take one gram of vitamin C the night before the urine examination,

Drink a half a litre of water 30 minutes before giving the sample

Collect the middle stream of urine

Collect at least 10 cc

Immediately send and process the sample (the best time is within 30 minutes of urination)

METHODS

10 to 20 ml of voided urine that is centrifuged at 1200 RPM per 12 minutes.

The supernatant is decanted, a drop of sediment is mixed with 0.1 % of saphranine and dropped onto the slide that is covered with coverslip and is ready for analysis. The second part of voided urine is prepared for cytospin sediment. A couple of drops are cytocentrifuged and 2 slides per sample are prepared for standard staining if additional techniques are not used.

In our laboratory Air-dried smears are stained by Papenheim and Haemacolor rapid staining and wet slides are fixed in 96% ethyl alcohol and Papanicolaou stained.

Analysis

Patient clinical data and pathology history are mandatory. The Information Technology department will give all to Pathology all the information in system, but in the request the urologist or the family doctor should add information about **therapy** that may affect morphology (radiation, chemotherapy, corticosteroids, hormones) or **operations**, and **radiological, biochemical and microbiological reports, as well as the urology history of the patient.**

Report

Each laboratory has its own way of describing the results of a urine cytology exam, and this is a matter of long, (futile) and exhausting discussions in panels of experts.

- **Unsatisfactory specimen.** Insufficient number of cells or wrong types of cells in urine sample. You may need to repeat the urine cytology test.
- **Negative.** This means no cancer cells were identified in your urine sample.
- **Atypical.** This indicates some abnormalities were found in the cells in your urine sample. But while the cells weren't normal, they weren't abnormal enough to be considered cancer.

- **Suspicious.** This term may indicate that urine cells were abnormal and may be cancerous. This term should be used with extreme care. It is not a waste-basket, but a category where enter those cases lacking strict, repeatable and
- **Positive.** A positive result indicates that cancer cells were found in your urine.

UC cannot be used alone to diagnose cancer. If atypical cells are detected, the patient very likely will undergo a series of invasive procedures, such as cystoscopy or computerized tomography.

MORPHOLOGY OF UC

THE CELLS	MORPHOLOGICAL CHARACTERISTICS
Urothelial cells	• desquamate from pelvis in kidney, ureters, bladder and urethra • variable shape and size (depends upon bladder extension)
Superficial or umbrella cells	• large flat and polygonal usually 20 – 30 μm (to over 100 μm) • mono and multinucleated (2 to 10 nuclei of variable sizes) • round to oval nucleus with powdery and finely granular regular chromatin, with small, prominent, basophilic chromocenters and eosinophilic, often multiple micronucleoli • abundant transparent cytoplasm may be dense, thin, vacuolated, with well-defined scalloped but smooth cytoplasmic borders
Intermediate or piriform cells	• smaller cells, round, elongated and whip-shaped in shape • round central nucleus with regular borders and powdery chromatin with single chromocenter mimicking nucleolus • well-defined cytoplasmic membrane, thickened, homogeneous cytoplasm
Basal or deep cells	• the smallest urothelial cells round in shape • pyknotic nucelus but regular in shape and follow the external shape of the cell • narrow rim of cytoplasm dense deeply stained
Squamous cells	• desquamate from trigone from bladder in female and from urethra in both sexes • non-keratinised squamous cells that respond to hormonal changes in both sexes • intermediate, superficial or anucleated cells
Columnar cells	• desquamate from trigone of bladder and urethra in both sexes • cells uniform in size and shape elongated with a terminal plate with attached short cilia, some may be goblet, mucus-producing • cytoplasm is translucent
Renal epithelial cells	• rarely seen in voided urine, often naked nuclei (very fragile cells), different in shape and size
From medulla	• cuboid or cylindric cells 15 – 60 μm, single or in clusters • well-defined, fine granular cytoplasm • nucleus is not pyknotic
From cortex	• oval or round cells 15 – 60 μm, usually single • ill-defined, fine granular cytoplasm • pyknotic nucleus
Erythrocytes	• qualitatively and semiquantitatively analysed
Glomerular	• point the damage of glomerulus • ring form or vesicular form or combination of these two morphological variants or ruined forms with grotesque shape
Non-glomerular	• derive from upper and lower urinary tract • double rim forms, spiked forms, discoid forms and creased forms with Mercedes star pattern in central zone

Classification

We use our own classification based on our experience and according to the literature data. In this list there are diseases that we shall discuss during the workshop:

1. **NORMAL URINE (NAD)**
2. **BENIGN LESIONS**
   a. Infections
      - Bacterial (Chlamydia trachomatis, mixed bacteria...)
      - Viral (polyoma virus, HPV...)
      - Parasitic (TV...)
      - Fungal
      - Tuberculosis
   b. Non-infectious inflammatory conditions
      - Malakoplakia
      - Eosinophilic cystitis
      - Renal parenchimal disease
         - Acute renal allograft rejection
      - Urothelial changes secondary to therapy
   c. Erythrocyturia (haematuria)
      - From the lower urotract
      - From the upper urotract
      - Glomerular lesion (glomerulopathia, glomerulonephritis)
      - Urolithiasis
3. **PREMALIGNANT LESIONS**
   a. Dyskaryosis
   b. Squamous metaplasia
4. SUSPICIOUS FOR MALIGNANCY
5. MALIGNANT TUMOURS
   a. Urothelial tumours
      - Papillary tumours
         - Papillary neoplasm of low malignant potential (PUNLMP) (grade I)
         - Low-grade papillary urothelial carcinoma (grade II)
         - High-grade papillary carcinoma (grade III)
      - Non-papillary tumours
         - Flat carcinoma in situ
         - Invasive carcinoma (high grade)
   b. Non-urothelial tumours
      - Squamous cell carcinoma
      - Adenocarcinoma
   c. Metastatic tumours
6. INADEQUATE
   a. Contamination with squamous cells in female
   b. Insufficient number of cells
7. NON-CLASSIFIED
   a. Reactive changes
   b. Polymorphia
   c. Atypia

NORMAL URINE according to the mode of collection

Voided urine

Sediment of the normal voided urine has few single epithelial cells: superficial or piriform urothelial cells usually with a single nucleus, squamous cells, columnar cells and rare and occasional tubular renal cells (<10 cells per 10 high-power fields), few non-epithelial cells: non-glomerular erythrocytes, neutrophilic granulocytes and lymphocytes and occasional non-cellular elements (hyaline and finely granular casts, mucus and corpora amylacea in men). Contamination of normal urine includes squamous cells and Döderline bacillus in females and seminal vesicle cells and spermatozoa in males.

Be careful: *seminal vesicle cells are very similar to malignant cells and very often the cause of misdiagnosis!*

Catheterised urine

Sediment of catheterised urine has many urothelial cells usually multinuclear with polymorphism in size and configuration: they can be single or in fragments: sheets, aggregates or 'pseudopapillary' clusters. Besides there are some columnar cells, many neutrophilic and eosinophilic granulocytes, many erythrocytes, moderate histiocytes, fibrin, debris, crystals and bacteria. There are usually no squamous cells and therefore it is the best kind of urine to be analysed for female when we get 3 times contamination in voided urine.

Be careful: *do not misdiagnose polymorphic urothelial cells with low-grade urothelial carcinoma!*

Ileal conduit urine

Sediment of ileal conduit urine `is hyper`cellular and markedly degenerated. There are numerous cells of intestinal epithelium, columnar cells with basally located nuclei, goblet cells and numerous inflammatory cells.

Be careful: *because ileal bladder is a container constructed surgically from a segment of small bowel after cystectomy: the presence of urothelial (transitional) cells is abnormal!*

Benign lesions

Infections

Infections can be caused by bacteria, viruses, fungi or parasites, can involve upper (pyelonephritis and ureteritis) and lower urinary tract (cystitis and urethritis) and can be acute or chronic.

During *bacterial infection* there are lots of cells in urine sediment: urothelial cells single or in sheets and small clusters with non-specific morphological changes (hyperchromatic and eccentric nucleus with uniform chromatin distribution, vacuolated or ill-defined amphophilic cytoplasm containing neutrophils), variable number of neutrophilic granulocytes (acute infection) or lymphocytes (chronic infection), non-glomerular erythrocytes (in native sediment: smooth in cystitis and dysmorphic in peylonephritis) and histiocytes on non-necrotic background. There are reactive urothelial cells: in toto larger than normal urothelial cells as well as nucleus and prominent nucleolus but nuclear-cytoplasmic ratio is normal. I n the background we can find bacterial rods and cocci that should be identified microbiologically.

Be careful: do not misdiagnose reactive urothelial cells with urothelial carcinoma!

The route of *tuberculosis* is opposite to bacterial cystitis: bladder TB occurs secondary to renal. We can see triad of granuloma: multinucleated cells of Langerhans' type, epithelioid cells, lymphocytes and plasma cells on the granular necrotic background. There are also reactive urothelial cells. We could sometimes identify Mycobacterium tuberculosis with Ziehl-Neelsen reaction.

Be careful: epithelioid granuloma could be caused by transurethral surgery with cauterisation or by immunotherapy if the patient was operated because of carcinoma and treated with BCG!

Viral infections are caused by DNA viruses that induce specific morphological changes of epithelial cells typical for each virus (polyomavirus, HSV, HPV, CMV, adenovirus type II, etc.). The identity of viruses should be established by immunological, virological and serological methods or by PCR.

Polyomavirus infection: tubular cells and urothelial cells from bladder and urethra are small, single with scanty basophilic cytoplasm and irregular hyperchromatic nucleus with basophilic large intranuclear inclusions in the

inclusion stage and enlarged nucleus with pale appearance and 'fishnet-stocking' pattern in the post-inclusion stage (decoy cells). Immunocytochemistry antigen to SV40 virus and anamnestic data about renal alograft transplantation could be helpful especially for postinclusion stage.

Be careful: decoy cells imitate malignant cells!

Human papillomavirus infections (mostly type 11 and 16) (HPV): koilocytes in voided urine from bladder and urethra but more often in urethral brushing of urethra in both sexes.

Be careful: in female patients do not mix it with HPV infection from genital tract!

In *parasitic infections* parasites are directly identified in the urinary smears (trichomonas, schistosomiasis, toxoplasmosis, enterobius vermicularis, etc.).

Trichomonas is rather rare but could cause urethritis and cystitis with marked acute inflammation with numerous neutrophilic granulocytes, squamous metaplastic cells, few urothelial cells, non-glomerular erythrocytes (in native sediment smooth) and trichomonas like a light gray pear-shaped protozoan without tail and with dark eccentric nucleus. Background is dirty but without necrosis.

Be careful: do not misdiagnose trichomonas as destroyed cells!

In *fungal infections* we can detect fungal spores and hyphal forms mixed with reactive urothelial cells and with marked acute inflammation. The most common is Candida albicans (spores or pseudohyphae), less common are histoplasma with intracellular yeasts in macrophages, actinomyces and aspergillus with septation and 45 degrees hyphae angle branching. They should be confirmed by microbiological culture.

Be careful: we are allowed to put the cytological diagnosis of fungal spores or hyphae, not the type of fungi!

Non-infectious inflammatory conditions

These include malakoplakia, sarcoidosis, eosinophilic cystitis, cystitis glandularis, cystitis cystica, renal parenchimal diseases like acute renal

allograft rejection and tubulointerstitial nephropathy, urothelial changes secondary to therapy, etc.

Malakoplakia is an uncommon granulomatous inflammation that usually affects middle-aged women. The lesion looks like a yellow plaque with a central depression (cystoscopy). Cytologically we can see many histiocytes with single or multiple spherical Michaelis-Guttmann bodies (orange inclusions) that are PAS and iron-positive. There are also few plasma cells and macrophages and lots of reactive urothelial cells on the granular background with necrotic debris.

Be careful: do not misdiagnose Michaelis-Guttmann bodies as ingested particles!

Eosinophilic cystitis is rare and usually associated with allergic diseases and bladder trauma, including transurethral or open bladder surgery (clinical data are important!). There are lots of eosinophilic granulocytes and non-glomerular erythrocytes (in native sediment smooth) mixed with neutrophilic granulocytes.

Be careful: sometimes there are more neutrophilic than eosinophilic granulocytes!

Cytology is very important for *acute renal allograft rejection* because it can predict allograft rejection in over 75% of patients before clinically recognisible rejection from 2 to 10 days after transplantation. Common cytological findings include increased cellularity with necrotic debris and pathological casts (epithelial and leukocytic). There are numerous non-glomerular erythrocytes (in native sediment dysmorphic), lots of reactive urothelial cells, very often decoy cells and numerous lymphocytes. When we compare the differential cell count of leukocytes in urine with the differential cell count with a simultaneously obtained sample of peripheral blood by conventional cytology, we can detect increased reactive lymphocytes and monocytes in blood and less than 55% neutrophilic granulocytes and more than 20% lymphocytes in urine as the sign of bad prognosis. These lymphocytes are T-lymphocytes with elevated CD8 supressor cells (identified by flow cytometry). There are >20 renal tubular cells per 10 high-power fields (large distal and small proximal tubular cells and endothelial cells). These cells have fine dusty granulations that are PAS- and alkaline phosphatase-positive (cytochemistry) and they express ICAM-1 and TNFalfa receptors (immunocytochemistry).

Be careful: it is not possible to distinguish acute renal allograft rejection from acute renal failure of other aetiology by cytology and therefore renal biopsy is recommended!

Urothelial changes secondary to therapy can be caused with intravesical and systemic chemotherapy, intravesical immunotherapy (BCG, interferon-ά), systemic hormones and corticosteroids therapy, radiation, etc.

Be careful: therapy-associated abnormalities remain the most common cause of false-positive reports and therefore we need patients' clinical history data!

Intravesical Bacillus Calmette-Guerin is the most effective treatment for non-invasive urothelial carcinoma and urine cytology is the most important tool for the follow-up. Cytological changes are not specific. There are lots of inflammatory cells, reactive urothelial cells, multinucleated giant cells, histiocytes, epithelioid cells and necrotic debris. It takes 4 or more months after termination of therapy to diagnose a cytological normal urine sediment.

Haematuria (erythrocyturia)

Haematuria can be essential, physiological or affected by anticoagulation therapy and radiation. It can be a part of a systemic disease such as leukaemia, Morbus Bechterew, haemophilia or can be caused by different pathological processes in upper and lower urinary tract such as tumours that can be strictly diagnosed by cytology. Cytology can point out some diseases like lithiasis, pyelonephritis or glomerulonephritis. Clinical data are important for cytological diagnosis of erythrocyturia caused by therapy or systemic disease or for physiological erythrocyturia.

Sometimes we can just point out the place of bleeding by qualitative and semiquantitative analysis of erythrocytes in native urine (erythrocyturia from upper urinary tract is defined by dysmorphic erythrocytes with spiked surface projections while eythrocyturia from lower is defined by smooth erythrocytes with normal shape of membrane).

Be careful: dysmorphic erythrocytes are not glomerular erythrocytes!

Semiquantitative analysis (urine native sediment)

- o Erythrocytes (E)
    - ▪ Differential cell count of erythrocytes establish the place of bleeding:
        - • > 70% smooth E - haematuria from the lower urinary tract
        - • > 70% dysmorphic E - haematuria from the upper urinary tract
        - • 50% dysmorphic E : 50% smooth E - mixed haematuria

Urolithiasis is characterised by polymorphism of the urothelial cells (alterations in the shapes and sizes with a degree of nuclear hyperchromasia) and sometimes crystalline deposits may be observed in the cytoplasm of these cells. There are lots of such urothelial cells, single or in clusters (compact three-dimensional balls or „papillary" clusters). Besides there are dyskaryotic and reactive urothelial cells and sqaumous metaplasia, numerous non-glomerular erythrocytes (in native sediment: usually both types – smooth and dysmorphic), many leukocytes and crystals (usually calcium oxalate). In stained cytospin sediments non-glomerular erythrocytes have different shapes: double-rim-forms with the thinness of the double rim, spiked forms of erythrocytes with the formation of spike-like projections on the cell surface usually in hypertonic urine that cause shrinkage of erythrocytes, discoid forms in hypotonic urine that cause enlargement of the erythrocytes and creased forms with bizarre creases that radiate in a „Mercedes star" pattern from a central clear zone of erythrocytes.

Be careful: do not misdiagnose polymorphic urothelial cells with low grade urothelial carcinoma!

Sometimes we can diagnose illnesses like glomerulopathia or glomerulonephritis by qualitative and semiquantitative analysis of erythrocytes in stained cytospin sediments. Significant features of glomerulonephritis are more than 80% glomerular erythrocytes in urine sediment.

- Semiquantitative analysis (in voided urine only)
-
    - Glomerular erythrocytes (GE) (Synonims: G1 dysmorphic erythrocyte or G1 erythrocyte)
        - Differential cell count of GE establish the damage of glomerulus:
            - < 20% GE - no glomerulopathia
            - 20 - 50% GE – possible glomerulopathia
            - 50 - 75% GE – possible glomerulonephritis
            - > 80% GE – glomerulonephritis
            -

Be careful: differentiate the percentage of glomerular and non-glomerular erythrocytes on 4 different fields in urine sediment, otherwise you can get the wrong differential cell count because glomerular erythrocytes bunch up together!

Glomerulonephritis is characterised by more than 80% glomerular erythrocytes of morphological types which we use in our practise: ring forms, vesicular forms, combinations of ring and vesicular forms and ruined forms (there are a variety of morphologic classifications!). The ring forms are the most common morphologic variant and are pathognomonic for glomerular haematuria. T he entire cell volume is arranged in a peripheral ring, giving the cell a 'doughnut' appearance. The vesicular forms have various degrees of membrane protrusions variable in their size and shape and may occur in isolation or combined with glomerular ring forms. The ruined forms often appear crushed and are severely distorted assuming grotesque shapes. Beside erythrocytes there are numerous renal tubular cells, lots of erythrocytic, coarsely granular and waxy casts and some urothelial cells (often reactive) in urine sediment. The cytological diagnosis of glomerulonephritis should be confirmed by electron microscopic analysis.

Be careful: use high magnification for analysis of erythrocytes, not misdiagnose ruined forms of glomerular erythrocytes with damaged erythrocytes; ruined forms have intact membrane!

PREMALIGNANT LESIONS

Squamous metaplasia

Squamous metaplasia is a pre-neoplastic lesion only if atypia is present (as in squamous cell carcinoma of bladder or in patients with prostatic carcinoma who are treated with hormonal therapy). It is characterised by keratinising metaplastic squamous cells with varying degrees of atypia of the cells and of the nucleus and by non-keratinising squamous cells without atypia.

Be careful: squamous metaplasia is not always the sign of premalignant lesions because we can detect it in chronic inflammation, prolonged lithiasis, vitamin D deficiency and in the patients with chronic urinary catheter (clinical data are important)!

Dyskaryosis

Dyskaryosis (synonyms are dysplasia or atypical cells) is not completely defined cytologically. These cells are precursors of carcinoma in situ and invasive urothelial carcinoma, which occurs in 15% of the patients within 5 years after cytological detection of dyskaryotic cells. Therefore we cytologically examine urine every 3 months in patients with dyskaryosis. We define dyskaryotic cells in urine sediment as increased basal cells uniform in shape, with irregular, hyperchromatic nucleus with slight variation in shape. Cytoplasm is scanty and there is slightly increased nuclear-cytoplasmic ratio. The cells shed singly or in papillary clusters.

Be careful: do not misdiagnose single dyskaryotic cells as single basal cells: use high magnification!

SUSPICIOUS FOR MALIGNANCY

The diagnosis of suspicious urothelial cells should rarely be used and only for those lesions with cytological changes similar to carcinoma (some diagnose them as atypical urothelial cells) and for those cases showing rare malignant cells that are insufficient for a diagnosis of malignant tumour. S uspicious cells are usually single with a large nucleus and irregular nucelar contours, hyperchromasia and irregular chromatin clumping and with slightly increased nuclear-cytoplasmic ratio. Such lesions must be carefully investigated and therefore we recommend repeating the cytological urine analysis within one month and suggest other medical examinations such as cystoscopy, CT, MR etc. The diagnosis of suspicious for malignancy encompasses most PUNLMPs, some low-grade papillary urothelial carcinomas and dysplastic flat lesions.

TUMOURS

According to the WHO histological classification of tumours of the urinary tract from 2004 the tumours are divided into urothelial tumours, squamous and glandular neoplasms, neuroendocrine, haematopoietic and lymphoid, melanocytic, mesenchimal, miscellaneous and metastatic tumours.

Urothelial carcinoma

95% of bladder tumours are urothelial carcinoma per se or in combination with squamous or glandular differentiation. At the time of initial diagnosis 75% of the patients have non-invasive urothelial carcinoma, 20% invasive and 5% have metastatic disease and most of them are papillary and multifocal. 60% of urothelial tumours are recurrent so when we once detect tumour in the patient, we shall control him/her throughout life. Urothelial tumours could be clasiffied as: papillary and non-papillary tumours. Papillary tumours are papilloma, papillary tumour grade I, papillary ca grade II and grade III. Non-papillary tumours are flat carcinoma in situ and invasive carcinoma.

Diagnosis of papilloma is strictly histological. There is an increased number of benign urothelial cells in papillary formation and a lot of erythrocytes in cytological sediment.

Papillary neoplasm of low malignant potential (PUNLMP) or papillary tumour grade I

PUNLMP is characterised by clean background, dyskaryotic cells in 3-dimensional clusters, lots of reactive urothelial cells and malignant urothelial cells in papillary clusters that can suggest the diagnosis. Nuclear-cytoplasmic ratio is slightly high, cytoplasm is thin and semitransparent and nuclei are round or oval with powdery chromatin, uniform "pencil-drawn" nuclear contours and small nucleoli. Cytology of the urinary sediment does not lend itself to the diagnosis of papillary tumours of low grade but cell blocks of urinary sediment enable the diagnosis of papillary tumour grade I.

Low-grade papillary urothelial carcinoma / papillary carcinoma grade II

These yield abnormal cells in urine specimens. The large clusters are mixed with a few small clusters and single atypical or malignant cells. The cells

are enlarged with a slightly irregular nuclear contour and moderate hyperchromasia with reduced nuclear transparency and prominent nucleoli. Cytoplasm is homogeneous and there is a moderately increased nuclear-cytplasmic ratio. There are some erythrocytes and leukocytes surrounding the tumour cells. Cytological diagnosis could be atypical cells or suspicious for malignancy or probably malignant low-grade papillary carcinoma. DNA pattern analysis by flow or image cytometry can help – aneuploid DNA is strongly suggestive of carcinoma. FISH (fluorescent in situ hybridisation) can also detect malignant cells.

High-grade papillary carcinoma or papillary carcinoma grade III

The diagnosis of is cytological. The background may be bloody, but necrosis is absent and that can help in distinguishing this tumour from invasive urothelial carcinoma. Cellularity is high with numerous tight clusters and single malignant cells. Malignant cells are pleomorphic, larger than normal. The cytoplasm ranges from homogeneous and scant to vacuolated and abundant and nuclei are pleomorphic and hyperchromatic with prominent nucleoli. Chromatin is clumped, irregular or vesicular and nuclear contour is irregular. Nuclear-cytoplasmic ratio is variable and "cannibalism" appears to be an indicator of high grade and invasiveness of urothelial carcinoma.

Carcinoma in situ, a flat urothelial carcinoma, is a precursor of invasive cancer. The diagnosis is cytological but we cannot distinguish carcinoma in situ from high-grade carcinoma. Background is without necrosis, there are few non-glomerular erythrocytes and inflammatory cells. Cellularity is variable. There is usually a monotonous population of medium-sized, or small malignant urothelial cells in small clusters, or single cell that we can cytologically suspect as carcinoma in situ. We ocasionally find few larger or bizzare malignant cells and in one third of the patients we can find pleomorphic cells that we cannot cytologically distinguish from high-grade carcinoma. They have scanty basophilic cytoplasm, nuclei are large, hyperchromatic with irregular contours, chromatin is coarse and irregular or pyknotic and nuclear-cytoplasmic ratio is markedly increased. It is important to stress that the cytological diagnosis of carcinoma in situ may remain unconfirmed for many years in the absence of an aggressive approach to bladder biopsies because it is not visible by cystoscopy.

Invasive non-papillary urothelial carcinoma is usually a high-grade carcinoma and the diagnosis is cytological. The prognosis for patients with

this carcinoma is poor and depends mainly on the clinical stage. The 5-year survival rate is less than 50% even with therapy. Background is dirty with marked inflammation, bleeding and necrosis. Pleomorphic malignant cells show variable sizes, variable nuclear-cytoplasmic ratio and anaplastic nuclei with irregular chromatin and large, irregular nucleoli. Squamous or glandular differentiation is common.

Some variants of urothelial carcinoma include sarcomatoid carcinoma that has a mixture of urothelial cells of high grade and a spindle cell component. Immunocytochemistry to cytokeratin, which is usually focally positive in sarcomatoid carcinoma and negative in sarcoma, is helpful in making the diagnosis. Another is lymphoma-like urothelial carcinoma, which is reactive with cytokeratin and carcinoembryonic antigen so they are positive while lymphoid markers are negative.

Non-urothelial carcinoma

Non-urothelial carcinomas are squamous cell carcinoma, adenocarcinoma that can be intestinal, signet-ring cell, mucinous or clear-cell and undifferentiated carcinoma that is very similar to small cell lung carcinoma.

Squamous cell carcinoma is frequently invasive and associated with keratinising squamous metaplasia. Predisposing factors in the development of this tumour are schistosomiasis, non-functioning bladders and renal transplants. Smears show pleomorphic malignant squamous cells, keratinisation and necrosis.

Be careful: in female patients a differential diagnosis of cervical carcinoma must be kept in mind!

Metastatic tumours

Metastatic tumours can directly spread from adjacent organs such as squamous carcinoma of the uterine cervix with squamous cells identical to primary squamous carcinoma of the bladder; endometrial carcinoma of the uterine corpus in postmenopausal female patients; colorectal carcinoma, which is relatively frequent and can be identified by immunocytochemistry using CEA; adenocarcinoma of prostate, which can be identified by immunocytochemistry using PSA.

Tumours can spread directly from the upper urinary tract, such as hypernephroma with fairly large cells with finely vacuolated and granular cytoplasm that can be identified by cytochemistry: PAS, PAS with amylase digestion and oil-red or Sudan III are positive.

Be careful: do not misdiagnose it as lipoblastic urothelial carcinoma.

Some metastatic tumours can be lymphatic or haematogeneous spread from distant sites such as melanoma - melanoma pigment can be detected by immunocytochemistry using HMB-45, Melan or S-100. Some carcinomas can spread in that way, such as bronchogenic or oesophageal squamous carcinoma, adenocarcinoma from the breast and gastrointestinal tract and small cell carcinoma of the lung, which is similar to small cell undifferentiated carcinoma of bladder. Immunocytochemistry can be helpful for the distinction these tumours: for example for lung adenocarcinoma we use TTF1 (it is positive). Haematolymphoid malignancies such as lymphoma, leukemia and multiple myeloma can also spread in this way: in these cases immunophenotyping by flow cytometry is very helpful.

Diagnostic value, biomarkers and ancillary tests

According to data from the literature, diagnostic accuracy of urine cytology of bladder carcinoma diagnosis ranges between 26% and 100%: it is low and variable for low-grade papillary neoplasms (accurate in 1/3 of the cases) and highly accurate for high-grade tumours including invasive carcinoma and carcinoma in situ (accurate in 3/4 of the cases). False-negative diagnoses range from 23% to 38% - so sensitivity is low, but specificity is high because false-positive diagnosis range only from 1.3% to 11.9% and are usually caused by urolithiasis, polyoma virus infection, seminal vesicle cells, instrumental affects cystoscopy included, radiation and chemotherapy.

The low sensitivity of conventional cytology may be solved by using biomarkers and ancillary tests that can improve the screening and detection of bladder carcinoma because they have better sensitivity than conventional cytology. Some of them are: bladder tumour antigen (BTA) test, nuclear matrix protein test – NMP22 immunoassay, hyaluronic acid-hyaluronidase (HA-HAase) test, cytokeratin 20 (CK20) immunocytochemical staining, flow cytometry (FCM), image cytometry, fluorescence in situ hybridisation (FISH) technique, telomeric repeat amplification protocol (TRAP) assay, etc. BTA test, NMP22 immunoassay and HA-Haase test have better sensitivity and lower specificity than conventional cytology.

Cytokeratin 20 (CK20) immunocytochemical staining is used to distinguish normal basal urothelial cells (negative CK20) versus dysplastic or malignant urothelial cells (positive CK20).

Be careful: benign superficial (umbrella) urothelial cells are CK20 positive!

Flow cytometry (FCM) is performed on urine cell suspension. It is used for DNA analysis (ploidy and proliferative activity) and discriminates high-grade (aneuploid) and low-grade (diploid) urothelial tumours. FCM has sensitivity 71% to 87%, specificity 80% to 92% and in combination with cytology sensitivity raises to 93% and specificity to 97%. It detects urothelial carcinoma 12 to 18 months earlier than cystoscopy and is a predictor of prognosis. It is excellent for monitoring therapy (aneuploidy means progression and diploidy regression of the tumour after therapy). FCM is used for immunophenotyping, for diagnosis of haematolymphoid malignancies and to predict alograft renal rejection.

Be careful: FCM requires large numbers of abnormal cells to give the accurate value (45% of voided urine give satisfactory result)!

Conclusion

Urinary cytology has a high specificity and positive predictive value for high-grade urothelial carcinomas but the well-known weakness in detection of low-grade urothelial carcinomas is still a challenge for cytologists. It has better specificity but lower sensitivity than ancillary tests and biomarkers which can improve screening and detection of bladder carcinoma especially low-grade but no single method or combination of analyses can yet be pointed out to replace cytology in the monitoring of bladder cancer. It is helpful in diagnosis of benign diseases of urinary tract and is cost-effective in avoiding unnecessary tests and analysis. Urinary cytology still remains the gold standard for the detection of urothelial carcinoma despite diagnostic limitations in general that are increased by the lack of a consensus classification system and high interobserver variability in the evaluation of cells with little atypia.

Choice from the literature

- WHO Classification of Tumours. Pathology and genetics of tumours of the urinary system and male genital organs. (ed. Eble JN, Suter G, Epstein JI, Seste rhenn IA) IARC Press, Lyon, 2004

- Koss LG. Koss' Diagnostic Cytology and its histopathologic bases. Volume I. Lippincott Williams and Wilkins, Philadelphia 2006; p.738-846

- Tetu B. Diagnosis of urothelial carcinoma from urine. Modern Pathol 2009;22:S53-S59

- Trutin Ostovic K, Bulimbasic S, Ljubanovic D, Galesic K, Savic I, Persec Z, Bartolin Z. Cytologic evaluation of hematuria. Acta Cytol 2010;54(3):412-413

- Cajulis RS, Haines GK, Frias-Hidvegi D, McVary K, Bacus JW. Cytology, flow cytometry, image analysis and interphase cytogenetics by fluorescence in situ hybridyzation in the diagnosis of transitional cell carcinoma in bladder washes: a comparative study. Diagn Cytopathol 1995;13:214-224

- Glatz K, Willi N, Glatz D, Barascud A, Grilli B, Herzog M, Dalquen P, Feichter G, Casser TC, Sulser T, Bubendorf L. An international telecytologic quiz on urinary cytology reveals educational deficits and absence of a commonly used classification system. Am J Clin Pathol 2006;126:294-301

- Sullivan PS, Chan JB, Levin MR, Rao J: Urine cytology and adjunct markers for detection and surveillance of bladder cancer. Am J Transl Res 2010;2(4):412-440

Breast Fine Needle Aspiration Cytology (FNAC)

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Introduction

In all settings, the main goal of breast FNA is to confirm benign or probably benign clinical and/or radiological findings in order to avoid unnecessary surgery or to give an unequivocal, preoperative diagnosis of malignancy in order to allow appropriate patient counselling and definitive clinical management.

Equivocal cytological diagnoses should lead to a diagnostic biopsy. For a breast FNA clinic to be successful, it is critical that the rates of inadequate and equivocal cytological diagnoses are low. The cytological findings should always be evaluated in conjunction with the clinical and radiological findings (triple assessment). Often FNA combined with radiology will determine patient management irrespective of clinical impression.

Discordant FNA and radiological results usually warrant a diagnostic biopsy. In specialised centers, sensitivity and specificity of breast FNA is around 90%, somewhat higher for palpable and non-palpable ultrasound-guided FNA than for stereotactic FNA.

The percentage of inadequate specimens should be less than 10%. The percentage of false negative diagnoses (FN) varies in the literature, but in specialised centres is usually less than 5%. The main cause of FN diagnoses is sampling error (SE). In about 70% of FN the target lesions is less than 1cm in size. False positive (FP) diagnoses are always interpretation errors (IE). They are highly undesirable, but in large volume institutions, they will occur from time to time in the process of evaluation of rare lesions, diagnostic pitfalls and look-alikes, such as some fibroadenomas with myoepithelial hyperplasia, complex sclerosing lesions and sclerosing adenosis. Most screening and other guidelines demand that the percentage of FP should be less than 1%.

Artifacts

As ultrasound localisation of breast lumps is adopted more widely, cell lysis by contamination by ultrasound transmission jelly poses a threat to cytological diagnosis. The effect of the jelly on cell morphology is

dramatic and, in experiments *in vitro*, varies with the length of exposure before fixation. The phenomenon is seen even in rapidly air-dried samples, presumably from the mixing of the jelly with the sample within the needle rather than on the slide. Initially, there is some swelling of the cell cytoplasm and nucleus. This can make benign cells more worrying when compared with uncontaminated controls. Cell swelling is closely followed by leakage of nuclear chromatin, then complete dissolution of cell structure to form granular basophilic material and, eventually, a basophilic 'soup'. The jelly may be misinterpreted as necrotic material or mucin. Radiologists regard ultrasound jelly as a bland substance. If made aware of its detrimental effects on aspirated cells, they can modify their technique to lessen the risk of contamination.

Cytological findings: the normal, mature breast

FNA from normal, adult breast tissue will contain variable amounts of ductal epithelial cell groups, small stromal fragments and fatty tissue. The epithelial cell yield is usually higher in younger age groups.

- Normal epithelial cells
- Small cohesive groups
- Monolayer sheets
- Occasional complete TDLU
- Oval nuclei with regular outlines, 8 – 10 μ m in diameter
- Inconspicuous nucleolus
- Evenly distributed chromatin
- Scanty cytoplasm
- Myoepithelial cells appear as ovoid, dense nuclei at the periphery of ductal sheets and groups
- Naked, bipolar (myoepithelial cell) nuclei in the background.

Cytological features of pregnancy-related changes

Acinar cells have abundant granular or vacuolated cytoplasm that is unusually fragile, frequently stripping away leaving naked nuclei in a granular 'dirty' background. The nuclei are large and round with active vesicular chromatin and a distinct large nucleolus. The nucleolus in lactating epithelial cells is larger than in most malignant breast tumours. However, the presence of lipid-laden secretory material in the background is a helpful feature.

- The aspirate may be moderately or markedly cellular

- The cells are single and well dispersed in a lipid rich foamy or granular background

- The cells and their nuclei are large; there is abundant vacuolated or wispy cytoplasm

- Bare nuclei are common. The nuclei are round and uniform with active granular or vesicular, but evenly distributed chromatin.

- Single prominent nucleoli

Diagnostic pitfalls: pregnancy-related changes

The main risk is that the low-power impression of a cellular aspirate of single, large cells is taken as evidence of malignancy when the pathologist is not aware of the pregnant or lactational state of the patient. The distinctive granular background and critical assessment of the nuclear features should prevent this error. It is important for the cytopathologist to be informed about pregnancy or lactation, when the aspirate is performed by others, to lessen the likelihood of interpretive errors; one must also remember that foci of lactational change can occur unassociated with pregnancy and generalised lactation.

Cytological findings: fat necrosis

- Foamy macrophages and multinucleate giant cells with foamy cytoplasm

- Small irregular groups of (reactive) histiocytic cells

- Fragments of normal as well as degenerate fatty tissue

- Variable numbers of other inflammatory cells but usually sparse

- Few if any epithelial cells

- Free lipid droplets, seen as empty spaces that may be surrounded by blood or as empty spaces in a granular background

- Granular background debris.

Diagnostic pitfalls: fat necrosis

- Aspirates from tuberculosis or other causes of panniculitis may be mistaken for fat necrosis

- Epithelioid cells in fat necrosis and granulation tissue can imitate carcinoma closely

- Vacuolated histiocytic cells may be mistaken for lobular carcinoma cells

- Fat necrosis is often found in the periphery of a tumour in areas where the carcinoma cells infiltrate fatty tissue. If the aspirated cells are from the periphery only, cells from the underlying tumour may not be seen.

Cytological findings: subareolar abscess

- Anucleate squames

- Multinucleate giant cells

- Macrophages

- Epithelium showing reactive atypia.

  Diagnostic pitfalls: subareolar abscess

  - Other inflammatory conditions including tuberculosis

  - Sometimes the reactive atypia in the epithelial component of the aspirate is such that it may be confused with a more significant lesion.

Cytological findings: fibrocystic change

- Scanty, often watery smear

- Low or moderate cellularity

- Apocrine cells either dominating the cellular picture or in variable numbers

- Macrophages and granular debris form microscopical cysts

- Sheets or fragments of ductal epithelium with bland nuclei arranged in a honeycomb pattern with admixed myoepithelial cells and dispersed bipolar nuclei

- Three-dimensional epithelial aggregates representing intraductal hyperplasia

- Fat or fibrous stroma in variable quantities.

  Diagnostic pitfalls: fibrocystic change

  - Benign fibrocystic change may mask an adjacent carcinoma

- A carcinoma infiltrating in an area of fibrocystic changes may be underdiagnosed as reactive/hyperplastic

- Apocrine carcinoma can occasionally be interpreted as benign if low grade

- Benign apocrine metaplasia may appear atypical when degenerate

- In florid hyperplasia there may be marked anisokaryosis that can be misinterpreted as atypia.

Cytological findings: fibroadenoma

- Moderate or high cellularity, but may be scanty in older or fibrotic lesions

- Cohesive sheets with an antler-like appearance containing recognisable myoepithelial cell nuclei

- Many naked bipolar cell nuclei in the background

- If apocrine or foamy cells are present they are few.

Diagnostic pitfalls: fibroadenoma

- As with all cellular benign lesions, the high cellularity should not be interpreted as suspicious

- Some fibroadenomas show reduced cellular cohesion and significant nuclear enlargement with anisonucleosis and prominent nucleoli that may risk a false positive diagnosis of carcinoma

- Occasionally, a very myxoid stroma, particularly when associated with over-spread dissociate epithelium, may mimic mucinous carcinoma

- Misdiagnosis of fibroadenoma is the commonest cause of false positive diagnoses, although these are rare

- Phyllodes tumour shares some cytological features with fibroadenoma.

Cytological findings: low-grade (benign and borderline) phyllodes tumour

- Cellular smears with occasional large sheets of benign epithelium

- Numerous plump, single stromal cells with little or moderate cellular pleomorphism. Occasionally bizarre, degenerative type nuclear abnormalities

- The prominence and number of bipolar cells are usually greater than in fibroadenomas

- Obvious stromal fragments, some large and with high cellularity

- Fragments composed entirely of bipolar cells containing pink or purple ground substance in MGG preparations.

- Occasional mitoses.

Diagnostic pitfalls: low-grade phyllodes tumour

- May be mistaken for a fibroadenoma

- Fibroblastic proliferative entities as fibromatosis and nodular fasciitis

- Spindle cell sarcomas

- Malignant myoepithelioma

- Bizarre degenerative nuclear atypia may be interpreted as a feature of malignancy.

Cytological findings: high-grade phyllodes tumour

- Properly taken aspirates are generally abundantly cellular

- Large atypical stromal cells, often in cohesive groups

- Sometimes overtly sarcomatous elements or fragments of densely cellular stroma with obvious mitoses are seen

- The epithelial content is variable but tends to be particularly sparse in frankly malignant phyllodes tumours. It is benign but may demonstrate hyperplasia and mild atypia.

Diagnostic pitfalls: high-grade malignant phyllodes tumour

- Obvious malignancy in a phyllodes tumour may be misdiagnosed as carcinoma, particularly metaplastic carcinoma.

Cytological findings: nipple adenoma, papilloma of the nipple ducts, erosive adenosis of the nipple, subareolar papillomatosis

- Moderate or high cellularity with a basic benign pattern

- Dispersed epithelial cells and small groups

- Little anisonucleosis, the uniform nuclei showing finely distributed chromatin and small nucleoli

- Occasional hyperchromatic nuclei possible

- Adenosquamous nests may be apparent

- Small amount of debris, inflammatory cells and siderophages may be a feature

- Apocrine cells may be present.

  Diagnostic pitfalls: nipple adenoma, papilloma of the nipple ducts, erosive adenosis of the nipple, subareolar papillomatosis

  - Clinically, may be mistaken for Paget's disease of the nipple

  - Low-grade carcinoma may be difficult to exclude except by local excision, which is in any case appropriate.

Cytological findings: intraductal/intracystic/sclerosing papilloma/papillomatosis

- Variable cellularity with a basic benign pattern

- The epithelial cells are often dispersed or in small groups

- Complex, folded three-dimensional epithelial aggregates

- Papillary clusters may be preserved

- Papillary stromal fragments may be present

- Small numbers of bipolar cells

- Apocrine cells may be present

- A small amount of debris and macrophages may be present.

  Diagnostic pitfalls: intraductal/intracystic/sclerosing papilloma/papillomatosis

  - When the lesions are cellular, differentiation from a well-differentiated papillary carcinoma may be difficult.

Cellular papillary lesions

Intracystic /intraductal papillary tumours may have growth patterns that range from a 'simple' benign papilloma to very cellular lesions with a marked epithelial proliferation and hyperplasia that resemble epithelial hyperplasia with and without atypia as well as fully diagnostic papillary intracystic carcinoma. Cytologically, these will present as cellular lesions with a moderate to distinct cellular/nuclear pleomorphism, papillary

fragments and fibrovascular stalks. Usually, the epithelial fragments are rather cohesive, but a population of single cells is almost always present as well. Apocrine cells, macrophages and intracystic debris are common.

As these lesions are so heterogeneous, it may be impossible to give a confident diagnosis of benign versus malignant lesion and they should all be excised. Most papillary carcinomas are low-grade and show a discrete atypia. However, they often have a monotonous cell appearance ('monoclonality'), whereas the benign, cellular papillomas reveal a more polymorphic ('polyclonal') cell population as is found in epithelial hyperplasia (see above).

Mucocele-like lesions (MLLs)

MLLs have been described as being associated with ductal hyperplasia, atypical ductal hyperplasia, intraductal carcinoma and invasive carcinoma. Pathological features are defined by the presence of cysts containing mucinous material that rupture, expelling secretions and epithelium into surrounding tissues. Cytologically, these lesions are characterized by abundant mucin and monolayer clusters or sheets of epithelial cells without nuclear atypia and none or few single cells. However, they may form a continuous spectrum ranging from benign to invasive mucinous carcinoma.

Cytological findings: MLLs

- Abundant mucin
- Scant to moderate cellularity with cohesive, monolayer clusters and sheets of epithelial cells
- No or up to moderate nuclear atypia
- No or only few single cells.

  Diagnostic pitfalls: MLLs

  - Risk of overdiagnosis as mucinous carcinoma

Malignant breast tumours

General criteria for malignancy

No single morphological feature can be relied upon to distinguish benign from malignant cells at any site. It is the complete picture with the 'pattern of the smear ', the nuclear and cytoplasmic details in conjunction with the radiological and clinical findings that leads to an accurate diagnosis.

Cellularity of the specimen

Most carcinomas produce aspirates with moderate or abundant cellularity. On the other hand, carcinomas with a scirrhous stroma, and those in which tumour density is low, notably many of the lobular carcinomas, yield a more scantily cellular specimen. Also, many benign lesions found in younger women may provide intensely cellular smears.

Dispersal of cells

Lack of cell-to-cell cohesion is a characteristic malignant feature, but it is not diagnostic of an invasive lesion. Most in situ lesions yield a variable amount of single cells. Also, some benign lesions may show discohesion, either genuinely or as an artifact due to too much pressure when smearing the material. Cellular discohesion is preserved in LBC preparations.

Absence of biphasic pattern with myoepithelial cells

In most invasive carcinomas, myoepithelial cell nuclei are missing, both in the background and in the periphery of the tumour cell groups and aggregates. Tubular carcinomas and some low-grade ductal carcinomas are an exception where a few myoepithelial cells may still be found. The naked myoepithelial nuclei in the background of the smear are also missing in in situ lesions. However, remnants of myoepithelial cells are found in the periphery of the cell groups and aggregates in a substantial proportion of non-high-grade ductal carcinoma in situ (DCIS) and even in some high-grade DCIS. Some carcinomatous aspirates contain a population of naked epithelial/tumour cell nuclei. Usually, these have obvious malignant features. However, some may be ovoid and therefore mistaken for bipolar cells. Careful attention to the quality of the chromatin and the appearance of the nucleoli should avoid this pitfall. Some malignant aspirates also contain hyperplastic but benign tissue resulting in a population of bipolar cells that distract attention from the population of malignant cells.

Nuclear size and pleomorphism

The size of nuclei in breast carcinoma cells may vary enormously, from one and a half to two times the diameter of a red blood cell (RBC) and even up to more than five times the size. Most low-grade carcinomas have nuclear sizes in the range of two to three times that of an RBC. Nuclear pleomorphism with varying size within a smear is characteristically found in grade 2 and grade 3 carcinomas. Nuclear size pleomorphism is less distinct in low-grade carcinomas and a few may appear deceptively monotonous ('monoclonal').

Nucleolar size and pleomorphism

Except for apocrine metaplastic cells, and secretory cells as in pregnancy and lactation, it is unusual for normal benign breast epithelial cells to have prominent or multiple nucleoli. Most low-grade carcinomas have small or indistinct nucleoli. Distinct or prominent/abnormal nucleoli are a feature of grade 2 and 3 carcinomas. However, a number of benign, but proliferative lesions, including fibroadenomas may present with a distinct nucleolus. In these cases the nucleus will have a uniform/smooth chromatin pattern. Some of the largest nucleoli are found in benign, lactating cells. Nucleoli, and the size of the nucleoli, are a feature of active cells. This is the case in most carcinomas, but the nucleoli as such are not a feature of malignancy. Atypical nucleoli with bizarre shapes and sharp edges in well-preserved cells are a feature of malignancy.

Nuclear membrane irregularity and extranuclear chromatin

Malignant nuclei almost invariably show nuclear profiles in which there are small indentations as folds, grooves and clefts or projections/buds. Box-shaped and angular nuclei are also suspicious. Extranuclear chromatin is very much a malignant feature but tends to be seen more commonly in higher grade carcinomas where diagnosis is not a problem.

Nuclear/cytoplasmic ratio and cytoplasmic features

This is of less help than at any other site in the body as normal breast epithelial cells can have scanty cytoplasm and carcinoma cells showing apocrine differentiation may have a great abundance. Intracytoplasmic lumina are an occasional feature of both lobular and ductal carcinoma cells but are only very rarely seen in benign breast epithelium.

Chromatin texture

In Pap-stained preparations, the appearance of a coarsely and unevenly stippled nucleus with variable but prominent chromocentres suggests malignancy. MGG-stained preparations give a more subtle, but no less characteristic appearance of a coarse 'rope-like' texture that, when marked, can give the impression of small nuclear holes.

Nuclear fragility

Malignant nuclei show a greater tendency to rupture under the physical pressure of being smeared. This tendency is variable and is seldom marked in breast aspirates unless excessive pressure is used. Nuclear rupture may, however, be a clue that dissociation of otherwise unremarkable epithelial cells may be artifactual rather than a sign of malignancy.

Mitotic figures

These are rarely seen in breast aspirates except those of high-grade ductal carcinomas. They can be a feature of benign lesions such as fibroepithelial neoplasms. Unless frequent and atypical, they should not be given a heavy diagnostic bias.

Contents of the background

Abundant necrotic material in an otherwise cellular smear is usually attributable to tumour necrosis. Most high-grade DCIS and a few grade 3 invasive ductal carcinomas show comedo type necrosis on the smears. Necrosis is thus not pathognomonic of invasion, and may even be more suggestive of a DCIS mass.

Cytological findings: invasive ductal carcinoma

- Varying cellularity from abundant to scanty
- The epithelial cells present as single cells, loose aggregates and cohesive groups often three-dimensional in appearance
- Varying cellular and nuclear atypia according to histological grade
- Cells may be vacuolated and occasional signet ring cells are seen
- Microcalcification is quite common
- Mitoses are uncommon, but may be seen in high-grade lesions
- Necrosis is not common.

High-grade DCIS

The cytological features of high-grade DCIS have been described in several papers in the literature. In a setting of mammographic microcalcifications without a tumour, the following features are characteristic of high nuclear grade DCIS: high-grade atypical carcinoma cells in three-dimensional, solid aggregates and as single cells, microcalcifications and comedo type necrotic material.

An eventual additional invasive component is, for all practical purposes, not recognisable in the smears. A radiological tumour with calcifications, will practically always be an invasive carcinoma which may or may not have a variable component of DCIS within it. Most high-grade DCIS will predominantly present as solid, three-dimensional epithelial aggregates. In some cases the aggregates may be cribriform. Micropapillary and true structures are occasionally seen. A few myoepithelial cells may be seen in the periphery of cell groups and aggregates.

Cytological findings: high-grade DCIS

- Solid or cribriform, three-dimensional aggregates of epithelial cells with high-grade nuclear atypia
- Occasionally micropapillary and true papillary structures as well as monolayer sheets
- Variable number of single cells; occasional cases may present as an almost exclusively single cell population
- Microcalcifications
- Comedo-type necrosis.

  Diagnostic pitfalls: high-grade DCIS

  - Some cases of grade 3 IDC may present with necrosis and microcalcification, but the clinical and radiological appearance will be that of a tumour
  - Microinvasion or invasive lesions that have not been seen radiologically will not be sampled, and about 20% of cases will have an invasive component in the surgical specimen
  - A large number of single cells is not a feature of invasion.

Low- and intermediate-grade DCIS

Low -grade and intermediate-grade (or non-high-grade DCIS) is more heterogeneous in appearance than high-grade DCIS both histologically and cytologically. The cytological features have been described in the literature, but the number of cases that form the basis of the criteria listed below, are far less than for the high-grade DCIS. The criteria overlap with criteria for epithelial hyperplasia both with and without atypia. Typical non-high-grade DCIS presents as very large, cohesive, three-dimensional and cribriform aggregates, as well as some solid aggregates, micropapillary groups and microcalcifications. Pure subtypes are virtually non-existent. The number of single cells may vary. In most cases, there are rather few, but here also, occasional cases may present with a dominant single cell pattern. The nuclear atypia is discrete to moderate with nuclear sizes one and a half to two times that of a red blood cell. A diagnosis or suggestion of a low-grade/non-high-grade DCIS should lead to a local excision only.

Cytological findings: low- and intermediate-grade DCIS

- Very large three-dimensional epithelial aggregates, cribriform and solid, often more cohesive than high-grade lesions

- Cell monotony

- Micropapillary groups

- True papillary structures

- Monolayer sheets

- Variable number of single cells; usually few, but occasional cases may present as an almost pure single cell population

- Microcalcifications

- Occasional comedo type necrosis

- Low to moderate nuclear atypia

- Recognisable myoepithelial cell nuclei in epithelial aggregates and sheets not rare (!).

  Diagnostic pitfalls: low- and intermediate-grade DCIS

  - The cytological criteria overlap with epithelial hyperplasia with and without atypia. A diagnosis or suggestion of a non-high-grade DCIS should only result in a local excision or core biopsy confirmation of the type and extent of the lesion, never mastectomy or removal of axillary lymph nodes

  - Some of the lesions may appear quite monotonous ('monoclonal') in contrast to benign, hyperplastic lesions ('polyclonal' cell pattern)

- Numerous single cells are not indicative of an invasive lesion
- Myoepithelial cell nuclei on the epithelial groups are no 'proof' of a benign lesion.

Cytological findings: papillary carcinoma (in situ and invasive)

- May be cystic on aspiration
- The cell material is usually abundant
- Epithelial cells are monotonous and appear 'clonal'
- Anisonucleosis, hyperchromasia, coarse chromatin and prominent nucleoli are uncommon
- Benign bipolar cells are absent from the background and myoepithelial cells are not seen within the groups
- Large papillary cell clusters forming arborising arrays bearing overlapping, palisaded cells on a fibrovascular core may be present as with papillomas
- Cells may be dispersed and the fibrovascular cores denuded
- The cells are often distinctly columnar in appearance, although this feature is shared with papillomas.

Diagnostic pitfalls: papillary carcinoma

- These lesions are usually low-grade and lack obvious cytological features of malignancy often leading to a suspicious diagnosis
- The diagnosis may be missed if bloodstained cyst fluid is discarded rather than sent for cytological examination, although in some cases it is not diagnostic. If there is any residual mass after drainage of cyst fluid, re-aspiration should be undertaken
- Distinction between a benign, proliferative papilloma and a papillary carcinoma may be difficult. In general the neoplastic lesion will appear 'monoclonal' with a distinct monotony of the cells, whereas the benign lesions are 'polyclonal' with a more polymorphic cell pattern

Cytological findings: invasive lobular carcinoma

- Scanty aspirates are common

- Tumour cells are well dispersed and mainly single or in small groups of two to five cells

- Cells are small, nuclei have an abnormal appearance with irregular outline

- Occasional single file 'chains', usually containing only three or four cells, may be seen

- Cytoplasm is scanty, with the nucleus eccentrically placed and some cells may contain an intracytoplasmic lumen with a signet-ring appearance

- Nuclei show slight but definite variation in size but tend to be round in shape. The chromatin is stippled but not coarse and the nucleolus inconspicuous in the classic type.

Diagnostic pitfalls: invasive lobular carcinoma

- Lobular carcinomas account for many of the false negative cases in most series. There are also problems in categorizing the tumour cytologically

- It is not always possible to distinguish lobular from ductal carcinomas. Cases with prominent intracytoplasmic lumina may be mistaken for signet ring carcinoma

- High-grade or 'pleomorphic' lobular carcinoma resembles high-grade ductal ca.

  Because of the diffusely infiltrative nature of these tumours it is common for lobular carcinoma cells to be seen along with a variety of benign epithelial changes.

Thyroid fine needle aspiration cytology

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Description

The workshop provides MGG and/or PAP stained smears illustrating thyroid cytopathology routine work and also including some less common cases. Histology from most cases in which surgery was the treatment option is included.

Educational objectives

- Training of the algorithm of thyroid FNAC smears evaluation.
- Development or improvement of differential diagnostic thinking (lecture) and practical slides reading.
- Demonstration of combined techniques advantages (MGG, PAP, cellblock sections, immunocytochemistry).
- Development of cytology-histopathology correlation understanding.
- Presentation of the thyroid FNAC structure in the Bethesda 2010 system.

Introduction

FNAC is a regular part of thyroid diagnostics. Frequently the cervical lymph nodes are aspirated as well.

Indications & contraindications of thyroid FNAC

Indications:

- Non functioning nodules ≥15mm, or any nodule with suspicious ultrasound features or high clinical risk of malignancy
- diffuse enlargement only if suspicious clinically or in ultrasound

Contraindications – none in ≥22G needle; in children local anaesthesia can be considered.

Possible complications:

- local haematoma
- local discomfort
- necrosis of the nodule aspirated (usually incomplete but interfering with subsequent histopathology investigation)
- vasovagal reaction (very rare)

Techniques, fixation, staining

- Usually 1-2 passes are recommended and are enough to obtain adequate material, but in rare cases more punctions may be needed.
- Both air-dried and wet-fixed smears are recommended.
- Evacuated liquid content should be sent as a whole and the wall of the cavity separately aspirated.
- In hypervascular lesions the technique without suction can yield less blood-diluted material.
- As a rule 2-6 smears are produced.
- If a neoplasia is highly suspected or confirmed by rapid on-site evaluation (ROSE) an extra puncture into a liquid fixative for processing a cellblock is recommended.
- Naked eye visible micro-fragments may be removed from smears and placed in a liquid fixative container (formalin or ethanol) for processing the cytoblock

- Routine combination of staining (May-Grünwald-Giemsa and PAP) enriches the information.
- Histochemistry or immunocytochemistry may be performed on PAP stained smears, liquid-based cytology (LBC) preparations or cellblock
- Molecular methods feasible, not routinely used yet in thyroid diagnostics
- Liquid materials processed via cytocentrifuge or LBC methods.

Basic principles of thyroid FNAC evaluation
- Strict evaluation of quantity and quality of the aspirated material (a diagnostic conclusion made on a non satisfactory sample is the main cause of diagnostic errors)
- Report should include:
  - Description of the smears background, cytoarchitecture of cell groups and tissue micro fragments, cellular features of individual cells.
  - Conclusion: diagnostic evaluation, or probability, ordered differential diagnosis as close as possible to the expected histopathology diagnosis.
  - Bethesda 2010 category following diagnostic conclusion.
  - Recommendation – optional part – usually if a more representative sample should be acquired or other clinical tests (e.g. serum calcitonin) performed.

An overview of thyroid FNAC diagnostic features (organized according to the Bethesda 2010 categories)

NONDIAGNOSTIC OR UNSATISFACTORY
- Less than 6 groups with at least 10 well readable cells
- Obscured with blood or debris.

- Cyst fluid with macrophages and blood only. Recommendation: correlate with clinical and US features. Repeat if US suspicious (risk of malignancy 4%).

BENIGN

This category covers hyperplastic and inflammatory processes.

Hyperplastic nodule (Benign follicular nodule, nodular goiter)

- Frequently low to moderate cellularity – a diagnostic feature rather than a suboptimal sample (provided the aspirator is experienced enough)
- Background :
  - colloid of variable density
  - admixture of fresh blood or degraded erythrocytes with macrophages
- Groups of regular follicular cells. Remnants of macro- or microfollicular architecture may be present
- Nuclei 6-7 microns, round, with regular margins. Polyploid nuclei possible.
- Cytoplasm poorly circumscribed weekly stained (except in oncocytes)
- Marginal resorption vacuoles in toxic goitre ("fire flame signal") – rarely manifest
- Oncocytes even without inflammatory features in middle-aged and old patients
- Possible admixtures:
  - intrathyroideal parathyroid body cells (difficult to distinguish without immunocytochemistry)
  - fatty tissue, connective tissue (repair in the cyst wall), skeletal muscle
  - respiratory epithelia
  - repair cells

Lymphocytic Thyroiditis and autoimmune (Hashimoto's) thyroiditis

- bloody background, few or no colloid
- reactive lymphocytes and plasma cells
- lympho-histiocytic aggregates (fragments of germinal centres)
- oncocytic cells – may be missing
- clusters of follicular cells– may be missing
- multinucleated and epithelioid histiocytes may be seen

Differential diagnosis

- o Warthin-like variant of PTC – nuclear features key for DD
- o Non Hodgkin's lymphoma in cases of florid lymphoid hyperplasia- flow cytometry may be needed for DD

Subacute granulomatous (de Quervain´s) thyroiditis

- Background debris
- Small follicular cells often with degenerative changes (paravacuolar granules)
- Mixed inflammatory infiltrate, frequently with PMNs.
- Single epithelioid cells and/or epithelioid granulomata
- Numerous, very large, multinucleated cells

Differential diagnosis:

- o Lymphocytic thyroiditis
- o Other systemic granulomatous conditions

Chronic sclerosing thyroiditis (Riedel)

- Rare, representative sample difficult to obtain
- Fibroblasts, myofibroblasts, fragments of fibrous tissue
- Occasional inflammatory cells, specially plasma cells. No necrosis.
- Multinucleated or epithelioid histiocytes may be seen
- Clonal studies show predominance of lambda to kappa light chains.

Differential diagnosis

- o Anaplastic carcinoma
- o Sarcoma

Acute thyroiditis

- Neutrophils dominate, often with degenerating features, some histiocytes
- Stains for organisms – Gram, MGG
- Granulation tissue

Differential diagnosis:

- o necrotizing malignancy – rule out the presence of neoplastic cells

FOLLICULAR LESION OF UNDETERMINED SIGNIFICANCE/ ATYPIA OF UNDETERMINED SIGNIFICANCE

This diagnostic category is reserved for specimens that contain cells (follicular, lymphoid or other) with architectural and/or nuclear atypia that is not sufficient to be classified as suspicious for a follicular neoplasm, suspicious for malignancy or malignant. On the other hand the atypia is more marked than can be attributed confidently to benign changes. A frequent cause for this uncertainty is a compromised specimen: poor cellularity, obscured by blood or excessive clotting. In general, the clinical approach to a nodule with an initial AUS/FLUS interpretation is a repeat biopsy after a reasonable interval (6 months in our practice), although in specific clinical settings other management options may be more appropriate.

Cytological features

- There is a prominent population of microfollicles in an aspirate that does not fulfil the criteria for Folicullar Neoplasm. This situation may arise when a predominance of microfollicles are seen in a sparsely cellular aspirate with scant colloid. Alternatively a more

prominent than usual population of microfollicles may occur in a moderately or high cellular sample, but the overall proportion of microfollicles is not sufficient for a diagnosis of Follicular Neoplasm.

- There is a predominance of Hurthle cells in a sparsely cellular aspirate with scant colloid.

- Interpretation of follicular cell atypia is hindered by artefacts, such as air-drying with cell enlargement or clotting artefact with apparent cellular crowding.

- A cellular sample is composed of a virtually exclusive population of Hurthle cells, yet the clinical setting suggests a benign Hurthle cell nodule (Lymphocytic thyroiditis or multinodular goiter).

- There are focal features suggestive of papillary carcinoma, including nuclear grooves, enlarged nuclei with pale chromatin and alteration in nuclear contour and shape in a predominantly benign-appearing sample.

FOLLICULAR NEOPLASM

This FNA diagnosis encompasses mainly *follicular adenomas* and *follicular carcinomas*, which cannot be distinguished on cytological grounds, requiring histopathology to assess invasion. Some highly cellular *hyperplastic nodules* may fall into this category as well as the *follicular variant of papillary carcinoma* (FVPTC) that frequently shows less evident nuclear features than the classic variant. Interobserver diagnostic variation even on the histopathology level is high.

Cytological features

- High cellularity

- Bloody background with sparse or no colloid
    - (FVPTC may show some dense colloid fragments)

- Microfollicular (acinar-like follicles) architecture, eventually with central colloid

- Regular nuclei
    - (FVPTC usually shows some nuclei irregularity and overlapping and occasional grooving that may be a clue for diagnosis)

Oncocytic variants of follicular neoplasms should be reported – the proportion is arbitrarily defined as 75% minimum

Differential diagnostic pitfalls concerning the above-mentioned:

- parathyroid hyperplastic or neoplastic nodule
- overlapping features of oncocytic tumours and medullary carcinoma

(Both can be solved with immunohistochemistry if available, or PTH assay on needle lavage fluid; if not, additional clinical tests – serum calcitonin - and non-morphology methods – radioimmunoassay from aspirate can help).

SUSPICIOUS FOR MALIGNANCY

A category bearing high probability of malignancy with lack of quantity and/or quality /spectrum of diagnostic features of a particular malignancy in question. This classification indicates the possibility of a slightly protracted and less aggressive solution. (hemithyroidectomy, intraoperative frozen section).

Most primary thyroid malignancies possess diagnostic features allowing FNA diagnosis in an optimal sample or suspicious in an suboptimal one. Exceptions are represented by follicular and oncocytic lesions.

Suspicious for papillary carcinoma

- nuclear features (see papillary carcinoma) less persuasive and frequently only in a small subpopulation of cells
- concomitancy of other pathology such as thyroiditis

Suspicious for medullary carcinoma

- many variants with different cytomorphology features
- lower frequency - experience in reading the slides
- Immunocytochemistry can be helpful. High serum calcitonin levels allow to transfer some suspicious cases for diagnostic cases of medullary carcinoma.

Suspicious for metastatic involvement

- history of previous malignancy not always available
- comparison with the histopathology of the presumed source very helpful if available

Suspicious for non-Hodgkin malignant lymphoma

- in patients with long-lasting lymphocytic thyroiditis and growth recorded an additional sample for flow cytometry can be helpful.

MALIGNANT

Papillary carcinoma

The commonest malignancy in thyroid – even sometimes allowing variant sub classification

Cytological criteria:

- High cellularity
- Background without colloid or with sparse heterogeneous colloid ("chewing-gum colloid")
- Monolayered sheets, papillary clusters with well defined borders, cell whorls
- Syncitial cytoplasm or dense blue with well defined membrane (metaplastic type)
- Nuclear features
  - enlargement
  - irregular shape, non round
  - overlapping
  - clear, homogeneous chromatin
  - grooving
  - pseudoinclusions

- Psammoma bodies
- Multinucleated cells frequently present

Pitfalls:

- microfollicular variant – usually diagnosed on FNA as follicular tumors or follicular lesion of undetermined significance.

- macrofollicular variant – may be diagnosed as colloid nodular goiter due to the abundance of colloid.

- cystic PTC– underdiagnosed if there are only few tumor cells present that may be misinterpreted as histiocytes. TTF-1 is useful in doubtful cases.

- oncocytic variant – nuclear diagnostic features of papillary carcinoma must be present. Differential diagnosis with encapsulated papillary oncocytic tumors and oncocytic follicular tumors may not be possible by FNA.

Differential diagnosis:

No one of the above listed criteria is pathognomonic of PTC and the diagnosis must be supported by the interpretation of the whole features present on smears.

- Intranuclear inclusions and grooving may also be seen in other lesions particularly in
  - o Oncocytic tumors
  - o Lymphocytic thyroiditis
  - o Hyalinising trabecular tumor
  - o Medullary carcinoma

- Papillary clusters may be absent in cases of PTC and may be seen in hyperplastic nodules and follicular tumors – nuclear features are the key for DD.

- Psamomma bodies are not frequently seen, except in the diffuse sclerosing variant, may be confused with inspissated colloid and may be present in benign conditions such as hyperplastic nodules and thyroiditis.

- TTF-1 and thyroglobulin immunocytochemical positivity useful when considering differential diagnosis of metastasis.

Medullary carcinoma

Less frequent - 5-10% of thyroid carcinomas – it is highly polymorphous, hence difficult to diagnose without active consideration of this possibility, it must be considered in every case with unusual FNA findings. Up to 50% of patients manifest with lymph node metastasis sometimes from an occult (micro) carcinoma. (Less frequently this behaviour is seen with papillary ca.)

The FNA features correspond to the spectrum of histopathology variants:

- background: blood , amyloid fragments that may be mistaken for colloid
- pattern: single cells, clusters, rosettes (similar to microfollicles)
- cell size and shape variable but usually monotonous within each tumor: large, small, spindle, plasmacytoid, oncocytoid…
- nuclei: some degree of anisokaryosis , may have bizarre nuclei; speckled chromatin specially in spindle cells, pseudo inclusions frequent, bi and multinucleation and prominent nucleoli particularly in plasmacytoid cells
- cytoplasmic granularity, particularly visible with MGG stain
- calcitonin-positive

Confirmation of high serum calcitonin levels is recommended in every diagnostic or suspicious FNA reports as the therapy is radical: total thyroidectomy plus cervical lymphadenectomy and the adequacy of initial surgical treatment has prognostic significance.

The main **differential diagnosis** is oncocytic follicular tumour; others include a broad spectrum of other malignancies, either primary like papillary carcinoma or hyalinising trabecular tumor, or secondary like melanoma or plasmacytoma

Immunocytochemistry is often essential for diagnosis so cellblock, LBC or PAP stained smears should be performed when this tumor is suspected. Always perform a panel of markers including thyroglobulin and calcitonin because some oncocytic tumors can present false positive staining for calcitonin in smears.

Poorly differentiated carcinoma

Tumours of follicular cell origin, they represent the link between the differentiated forms of follicular and papillary neoplasms and anaplastic

carcinoma. The cytologic features overlap with follicular neoplasia . The main cytological findings are:

- high cellularity

- bloody background, no colloid
- solid cell nests (insular architecture) and single cells
- monomorphous cell population, usually small cells
- dark nuclei, sparse cytoplasm
- mitosis and necrosis frequently present.

The WHO classification states that the final diagnosis of poorly differentiated carcinoma requires histopathology investigation.

Undifferentiated (anaplastic) carcinoma

Highly aggressive tumor, presenting as a rapidly growing, locally progressive hard mass in the neck of elderly patients frequently accompanied by stridor, hoarseness or dysphagia. FNA is particularly useful as the main clinical differential diagnosis is lymphoma which is treatable, whereas anaplastic carcinoma is inoperable in most cases as well as not sensitive to radiotherapy or chemotherapy.

Variable cytology features:
- high cellularity.
- bloody or necrosis background
- spindle, polymorphous, plasmacytoid or rhabdoid cell population
- admixture of squamous component and giant cells common
- high mitotic rate,including atypical mitoses
- thyroglobulin, calcitonin and cytokeratin frequently negative
- cellblock useful

Differential diagnosis:
- Sarcomas, Metastasis, Lymphomas.

If smears are entirely composed of squamous cells the differential diagnosis between anaplastic carcinoma with a predominant squamous component, thyroid squamous cell carcinoma or secondary squamous cell carcinoma may not be possible either on FNA samples.

Metastatic tumours

Metastases from distant organs are unusual the most frequent being from kidney, lung, melanoma, breast, bowel

Local progression from pharynx, larynx, oesophagus or metastatic lymph nodes is more common

Diagnostic clues

- presence of a mixture of normal follicular cells and tumor cells
- tumor cytological pattern unusual for a thyroid malignancy
- history of previous malignancy helpful
- comparison with the histopathology of anamnestic malignancy
- immunocytochemistry usually needed for diagnosis

Malignant thyroid lymphomas

- mostly low grade B cell lymphoma - extranodal marginal zone lymphomas EMZL (MALT) - with clinical history of long-lasting Hashimoto's thyroiditis. Flow cytometry very useful for differential diagnosis
- less frequently DLBCL - rapid growth mimicking anaplastic carcinoma

SOME NOTES OF ANCILLARY TECHNIQUES ON THYROID FNA

Ancillary techniques have been proposed to improve diagnostic accuracy in the group of indetermine cytology: AUS/FLUS and suspicious for malignancy. ICC markers, such as CK19, galectin-3, and human mesothelial cell 1 (HBME-1), are used most often. The presence of these markers can be detected easily in smears, methanol-fixed thin layer preparations, and cell blocks.

CK19 is expressed strongly and diffusely in PTC, however, it is not specific and stains follicular cells of lymphocytic thyroiditis and of follicular adenomas or carcinomas.

Galectin-3, a protein involved in cell-to-cell and cell-to-matrix interaction, is considered a marker of malignancy in thyroid follicular lesions. Galectin-3 is positive in most PTCs and anaplastic thyroid carcinomas; however false-positive results can be obtained in lymphocytic thyroiditis, follicular adenomas or carcinomas, and medullary carcinomas.

Most PTCs have diffuse, positive immunostaining for HBME-1. This positivity is not exclusive to PTCs and, thus, does not specifically indicate

PTC. The use of a panel with galectin-3 and HBME-1 amplifies sensitivity and specificity, having a high negative predictive value for malignancy in indeterminate cytology when both markers are negative.

The finding that some thyroid lesions are associated with specific genetic alterations raises the possibility of improving FNA cytology diagnoses with access to molecular techniques.

PTCs can display non-overlapping mutations of the genes BRAF, RET and RAS in 46-75%, 3-85%, and 0-21% of cases, respectively. Although the prevalence of these mutations varies among the published series, their presence is very specific and indicates the existence of malignancy.

The follicular variant of PTC has a mutational framework that differs slightly from other PTCs. A distinct BRAF mutation is observed ([K601E]) in 7% of tumors, RAS mutations are more frequent (approximately 25% of tumors), and they can have a PAX8-peroxisome proliferator-activated receptor gamma (PPARgama) mutation (38% of tumors).

Follicular neoplasia has a high prevalence of RAS and PAX8-PPARgama gene mutations (33% vs 45%, respectively, in follicular adenomas vs follicular carcinomas). Characteristic follicular neoplasia mutations are less specific than those described in PTCs and do not necessarily point to malignancy. However, even using a panel of markers to detect BRAF, RAS, PAX8/PPAR, and RET/PTC mutations those were found in 16% of cases. These genetic markers have high specificity and a high positive predictive value for malignancy but the sensitivity is low.

Recently, a RNA-based test where the cells are aspirated using two passes and placed into a preservative solution were developed (Afirma gene expression classifier [AGEC], Veracyte, San Francisco, CA) for the preoperative identification of benign thyroid nodules whose cytology is indeterminate. A large prospective validation study of the AGEC showed an overall NPV of 94%–95% for AUS/FLUS and Follicular/Hurthle Cell Neoplasm subtypes. The NPV for cytology that proved Suspicious for Malignancy was lower, however (85%), indicating that the AGEC should not be used to avoid a surgery in this cytological subtype. In thyroid aspirates other differential diagnoses need to be considered.

Medullary thyroid carcinomas (MTCs) express calcitonin, carcinoembryonic antigen (CEA), synaptophysin, and chromogranin. Calcitonin is more specific than CEA; however, some MTCs can be only focally positive or even negative for this marker. Calcitonin is lost with dedifferentiation of MTCs, whereas CEA expression is retained. False-positive results have been demonstrated in cytological material. MTC is sporadic in 75% of patients and, in 25%, can be associated with inherited endocrine syndromes. Regardless of the clinical context involved, RET

mutations frequently are associated with MTC. MTCs associated with inherited endocrine syndromes have specific RET mutations that help to identify these patients. This knowledge will guide clinicians in the appropriate management of patients.

References

1. Alexander E, Kennedy G, Baloch Z et al. Preoperative diagnosis of benign thyroid nodules with indeterminate cytology. New Engl J med 2012; 367: 705-715.

2. Ali SZ, Cibas ES eds. The Bethesda system for reporting thyroid cytopathology. Definitions, criteria and explanatory notes. Springer , 2010, 171 s.

3. Cochand-Priollet B, Schmitt FC, Totsch M, Vielh P. The Bethesda Terminology for Reporting Thyroid Cytopathology: from theory to practice in Europe. Acta Cytologica 2011; 55: 507-511.

4. Dušková J, Tretiník P.: Papillary thyroid carcinoma: an easy fine needle aspiration biopsy diagnosis? Acta Cytol. 2011;55:120-30.

5. Fadda G, Rossi ED, Raffaelli M et al. Follicular thyroid neoplasms can be classified as low- and high-risk according to HBME-1 and Galectin-3 expression on liquid-based fine-needle cytology. Eur J Endocrinol 2011; 165: 447-453.

6. Kocjan G, Cochand-Priollet P, de Agustin P et al. Diagnostic terminology for reporting thyroid fine needle aspiration cytology: European Federation of Cytology Societies thyroid working party symposium, Lisbon 2009. Cytopathology 2010; 21: 86-92.

7. Nikiforov YE, Ohori NP, Hodak SP, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. J Clin Endocrinol Metab 2011; 96:3390-7.

8. Schmitt F, Barroca H. Role of ancillary studies in fine-needle aspiration from selected tumors. Cancer Cytopathol 2012; 120:145—60.

9. The Art and Science of Cytopathology, *2d ed.* In: Richard M DeMay. American Society for Clinical Pathology Press eds. 2012, ss 840-963.

10. Thyroid Cytopathology. An Atlas and Text. In: Sudha R Kini. Lippincott Williams&Wilkins/Wolter Kluwer eds. Philadelphia. 2008

11. Thyroid Cytopathology, *2d ed*. In: Douglas P Clark, William C Faquin. In: Essentials in Cytopathology Series. Springer, New York eds. 2010

12. Tumours of the Thyroid and Parathyroid. In: Ronald A. DeLellis, Ricardo V. Lloyd, Philipp U. Heitz, Charis Eng eds.: Pathology and Genetics of Tumours of Endocrine Organs, IARC Press , Lyon, 2004, ss. 49-134.

Serous effusions including immunocytochemistry
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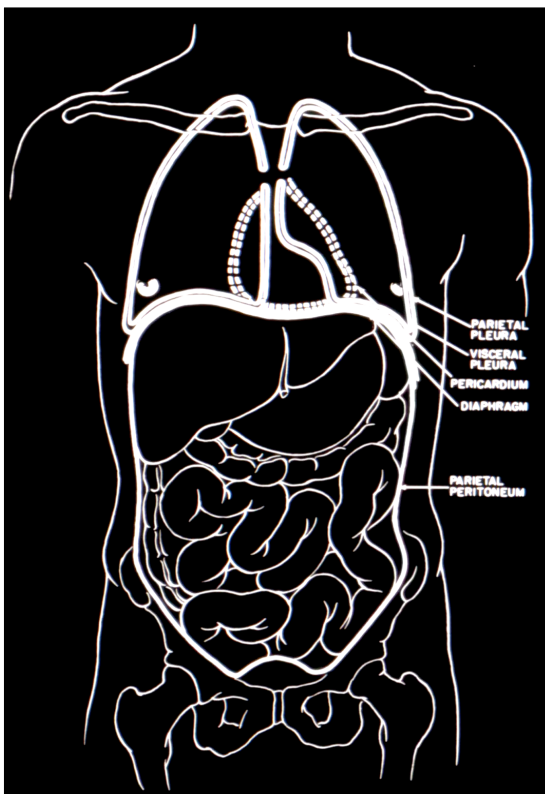
Main physioplogic characteristics

➤ Selective barrier (cells and fluid)

➤ Contains approximately 10 ml of liquid

➤ Covered by a single layer of mesothelial cells

➤ Cell diameter: 18 to 40μm.

➤ Long microvilli: 3μm X 0.1 M

➤ Microvilli of mesothelial cells: possible regulatory action protein: turnover - 0.7 mL / hour

➤ Small molecules (<4 nm diameter): diffusion through intercellular spaces and

➤ Large molecules (> 50nm diameter): pinocytosis - transcellular transport

➤ Visceral serosa is continuous; Serous parietal: STOMATAS discontinuity between the mesothelial cells (2 to 12μm in diameter)

Pathophysiological causes of effusions

1 - Increased hydrostatic pressure

2 - Decreased plasma oncotic pressure

3 - Retention of sodium

4 - Lymphatic obstruction

5 - Increased vascular permeability

Equation of Starling

$$F = K\,[(P_{cap} - P_{pl}) - X\,(\pi_{cap} - \pi_{pl})]$$
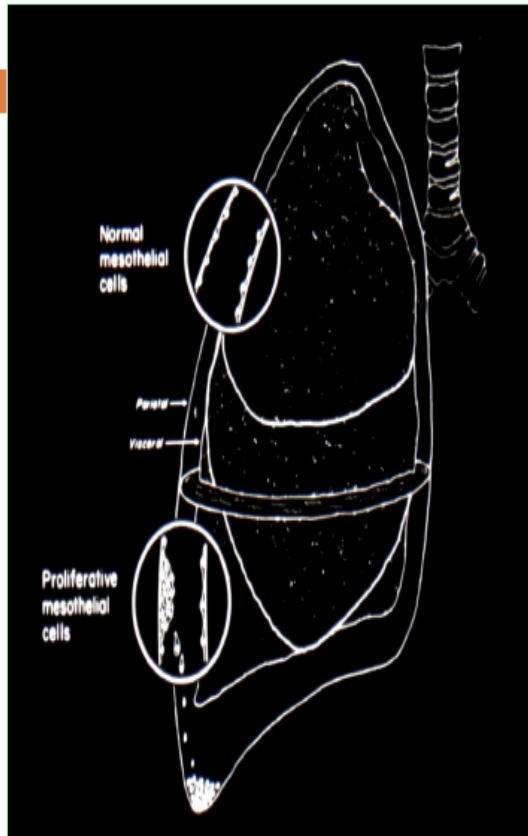
F = ratio of movement of fluids

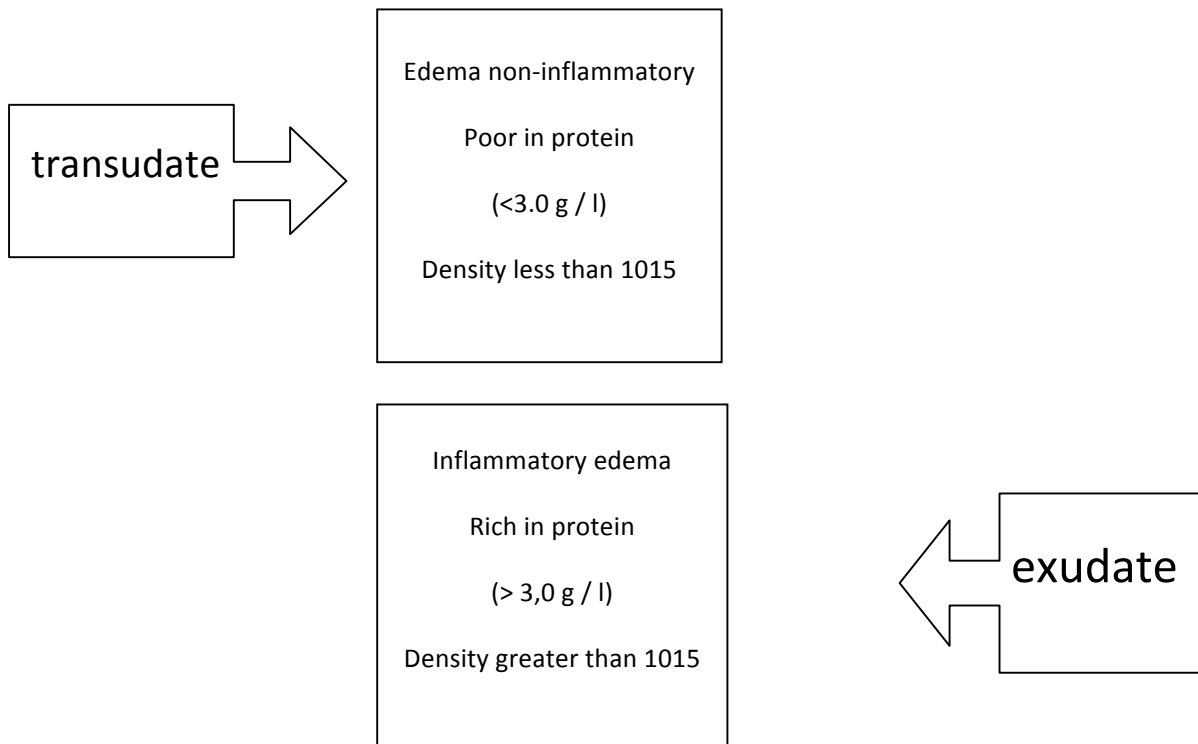P and π = hydrostatic and oncotic pressure, respectively

Filtration coefficient K =

X = osmotic reflection coefficient for protein (= 0.9)

cap = capillary

pl = pleural space

Normal mesothelial cells

Parietal

Visceral

Proliferative mesothelial cells

```
┌──────────────┐        ┌─────────────────────────────┐
│              │        │   Edema non-inflammatory    │
│  transudate  │───────▶│                             │
│              │        │     Poor in protein         │
└──────────────┘        │                             │
                        │        (<3.0 g / l)         │
                        │                             │
                        │   Density less than 1015    │
                        └─────────────────────────────┘

                        ┌─────────────────────────────┐
                        │     Inflammatory edema      │
                        │                             │        ┌──────────────┐
                        │      Rich in protein        │        │              │
                        │                             │◀───────│   exudate    │
                        │        (> 3,0 g / l)        │        │              │
                        │                             │        └──────────────┘
                        │  Density greater than 1015  │
                        └─────────────────────────────┘
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Transudate   **Exudate**

┌──────────────────────────────┐   ┌──────────────────────────────┐
│ 1 - Cholesterol: <60mg/dL   │   │ 1 - Cholesterol> 60mg/dL   │
│   │   │   │
│ 2 - DHL: <200 U / L   │   │ 2 - DHL:> 200 U / L   │
│   │   │   │
│ 3 – Pleural effusion / serum │   │ 3 - Pleural effusion / serum │
│   │   │   │
│ 3.1-[] Protein <0.5   │   │ 3.1-[] Protein:> 0.5   │
│   │   │   │
│ 3.2-DHL: <0.6   │   │ 3.2-DHL:> 0.6   │
│   │   │   │
│ 3.3-Bilirubin: <0.6   │   │ 3.3-bilirubin:> 0.6   │
│   │   │   │
│ 3.4-Cholesterol <0.3   │   │ 3.4-Cholesterol> 0.3   │
└──────────────────────────────┘   └──────────────────────────────┘

Features cytological the mesothelium normal

- ☐ Flat polygonal cells
- ☐ Single layer
- ☐ Cuboidal appearance in effusions
- ☐ Nucleus small, round or oval / central or peripheral
- ☐ Homogeneous chromatin
- ☐ Visible nucleoli
- ☐ Clear cytoplasm / smooth edges and well defined

General characteristics of reactive mesothelium

- ☐ Hyperplasia / hypertrophy
- ☐ Arrangements Papiliformes / acinar - clear spaces
- ☐ Irregular distribution of chromatin
- ☐ Dyskaryosis
- ☐ Pleomorphism
- ☐ Prominent nucleoli
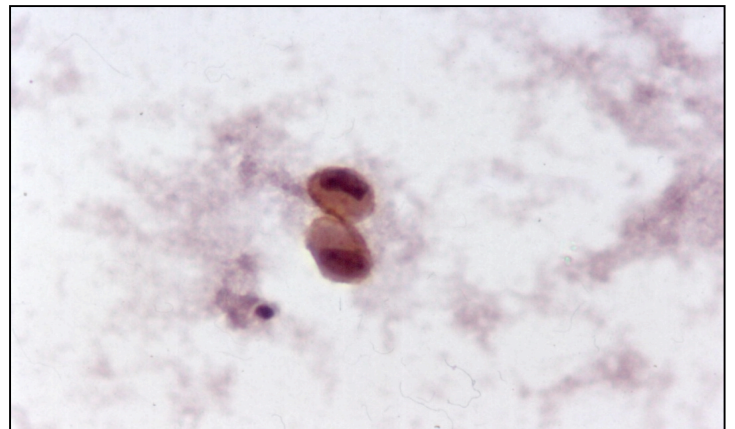- ☐ Multinucleation / Mitosis

Cytochemical features of the non-neoplastic

- Negative for PAS (periodic acid-Schiff): absence of neutral mucoproteins
- Positive for Alcian Blue-and Colloidal Iron: presence of acidic mucoproteins
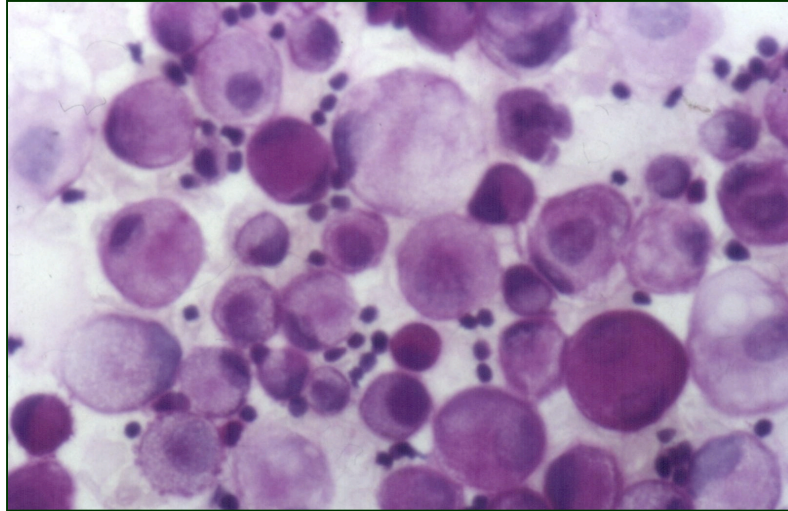
Pleural Fluid - Breast cancer. PAS 400X

Immunocytochemical features

Normal mesothelial cells: Ca125 Positive

POSITIVE (weakly) or NEGATIVE : CEA & EMA

NEGATIVE: LEU-MI (CD 15, a marker of leukocytes)

POSITIVE: vimentin. EPITHELIAL DIFFERENTIATION: vimentin inhibited

POSITIVE CK 7, 8, 18 & 19. DIFFERENTIATION fusiform: CK inhibited

POSITIVE: HAM-56 (marker of macrophages)

POSITIVE (occasionally): Ca-125 (ovarian epithelium):

POSITIVE: Thrombomodulin, Calretinin, HBME-1, mesothelin, N-Cadherin.

GENERAL CHARACTERISTICS OF MALIGNANCY

- ☐ Dyskaryosis
- ☐ Atypical mitosis
- ☐ Coarse & irregular chromatin
- ☐ Prominent nucleoli
- ☐ Arrangements: papillae, acini & ball cells
- ☐ Bi and / or multinucleation
- ☐ Pleomorphism
- ☐ Cellularity

Cytomorphological features of mesotheliomas

- ☐ Large and small groups coexist
- ☐ Cells isolated from coarse chromatin
- ☐ High cellularity
- ☐ Increased cell size
- ☐ Visible nucleoli
- ☐ Vacuolated cells
- ☐ Psammoma bodies
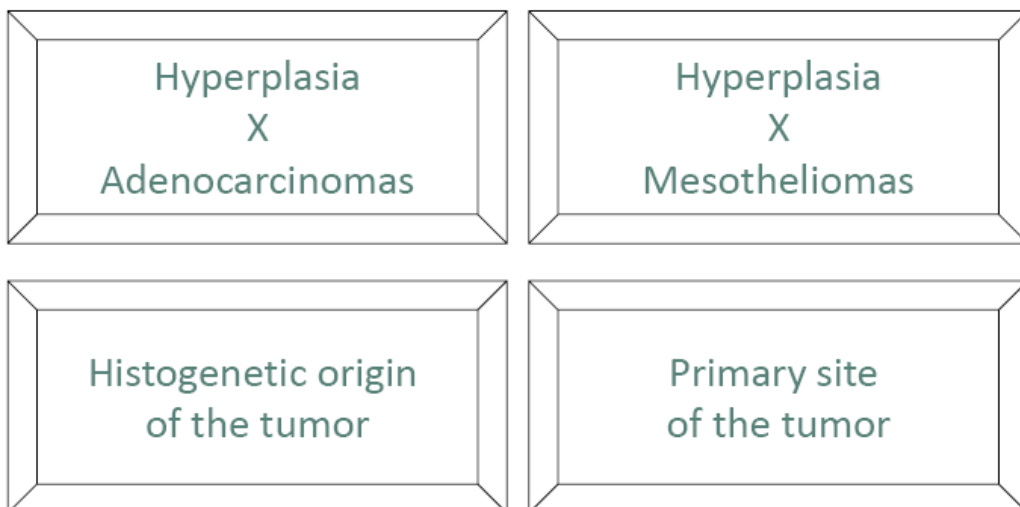- ☐ Numerous microvilli and intermediate filaments (Electronic microscopy)

Main Characteristics of Melanoma

- Melanin pigment (brownish)
- Absence of melanin (amelanotic)
- Round and abundant cytoplasm
- Vacuolated cytoplasm mimicking adenocarcinoma (signet ring pattern)
- Intra-nuclear inclusion

Cytological nightmares

How Immunocytochemistry helps desperate cytologists

Frequent issues in serous effusions cytology: Immunocytochemistry can help!

Hyperplasia X Adenocarcinomas	Hyperplasia X Mesotheliomas
Histogenetic origin of the tumor	Primary site of the tumor

What we must consider to select a proper IPX?

- Differentiation patterns

- Speculate about the potential primary site

- Cell secretion products

- Prognostic evaluation

- Mutation?

- Upregulation of a protein

Immunocytochemical panel to differentiate Mesothelioma and Adenocarcinoma		
Antibody	Mesothelioma	Carcinoma
Calretinin	Positive	Negative
CK 5/6	Positive	Negative
B72.3	Negative	Positive
BerEP4	Negative	Positive
CD 15	Negative	Positive

Immunocytochemical panel for adenocarcinoma differentiation

- Breast: CK7 +, CK20 -, CEA +, BRST2 +, Lactoferrin +, Vimentin -, ER +, PR +

- Ovary (serous): CK7 +, CK20 -, CEA -, Vimentin +, Ca125 +

- Colon: CK7 -, CK20 +, Vimentin -, CEA +, CA19.9 +

- Pancreas/ Bililary tract: CK7 +, CK20 +/-, Vimentin +/-, CEA +, CA19.9 +

- Stomach: CK7 +/-, CK20 +/-, Vimentin -, CEA +, CA19.9 +

- Lung: CK7 +, CK20 -, CEA +, Vimentin -, TTF-1 (nuclear) +

- Prostate: CK7 -, CK20 -, Vimentin +/-, CEA -, PSA +, PSAP +

Types of positive immunoreaction

1- PCNA...................................	Nuclear
2- Ki-67....................................	Nuclear
3- p53......................................	Nuclear
4- C-erbB2...............................	Membrane (citoplasm)
5- p27^{Kip1}..............................	Nuclear
6- CD34....................................	Blood Vessels
7- Antigens H & Y................	Blood Vessels

Take home messages

- Define area
- Random counting
- Count Positive Cells

- Define number of cells to be counted
- *Define Cut off*
- Define report contents