



Review

# The International System for Reporting Serous Fluid Cytopathology: How to Incorporate Molecular Data in Cytopathology Reports

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**Abstract:** Serous effusion cytology is widely employed in the initial evaluation of the etiology of effusions with a high diagnostic sensitivity. To standardize practices, The International System for Reporting Serous Fluid Cytology (TIS) was developed following best international practices, the most up-to-date literature, and expert consensus. In the context of this system, ancillary techniques play an important role. Besides defining basic principles in laboratory specimen handling, adequacy criteria, and a standardized reporting terminology with five diagnostic categories, TIS provides an actionable framework for using immunohistochemical and molecular testing in effusion samples, namely, in atypical, suspicious of malignant samples. For diagnostic purposes, these tests may be employed to distinguish between a primary and secondary neoplasm, to confirm a diagnosis of malignant mesothelioma vs. reactive mesothelial hyperplasia, and to correctly classify and determine the primary location of a metastasis. Theranostic molecular tests may also be used for these samples to evaluate potential therapeutic targets. Pathologists play a central role in guiding this process by determining adequacy and selecting appropriate ancillary tests. The activity in this area of research should increase in the near future as new therapeutic targets are discovered and new drugs enter the clinical practice.

**Keywords:** serous fluids; effusion; international system; molecular pathology; cytology; standardization



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## 1. Introduction

Serous effusions develop in both neoplastic and non-neoplastic pathological states [1]. Serous effusion cytology is widely employed in the initial evaluation of the etiology of effusions with a high diagnostic sensitivity [2]. In the context of malignancy, effusion samples provide adequate material for molecular testing, extracted from neoplastic cells as well as supernatant fluid [3,4].

The information extracted from these samples is not uniform between laboratories, as different collection and preparation techniques are employed, and the level of experience of cytopathologists also varies greatly. This issue has been successfully tackled in several areas of cytopathology through the development of standardized reporting systems [5–10]. In the context of serous effusions, The International System for Reporting Serous Fluid Cytology (TIS) was developed as part of a project sponsored by the International Academy of Cytology (IAC) and American Society of Cytopathology (ASC) [11,12]. International reporting practices were surveyed before the project, and the system is based on the most up-to-date literature. TIS defines five diagnostic categories: non-diagnostic (ND),

negative for malignancy (NFM), atypia of undetermined significance (AUS), suspicious for malignancy (SFM), and malignant (MAL). Each of these is characterized by strict diagnostic criteria and a well-defined risk of malignancy (ROM). The hope is that the implementation of this system will lead to better interobserver agreement and patient management [13]. In the context of TIS, ancillary techniques play an important role. In AUS and SFM categories, they may be used to make a more definitive diagnosis; in the context of the MAL category, ancillary testing may be used to establish the primary site of a metastasis, correctly classify an hematolymphoid malignancy or to evaluate prognostic or theranostic markers [14].

In this paper, we briefly summarise the nomenclature and criteria of the TIS diagnostic categories and review the proposed use of molecular tests within each category.

## 2. TIS—A Brief Overview

TIS aims to improve the diagnostic yield of serous effusion cytology through its diagnostic categories with well-defined ROMs, which, when coupled with correct sample handling, should lead to an increase in interobserver agreement and enable better, evidence driven, clinical management [12,14].

The aforementioned five diagnostic categories (ND, NFM, AUS, SFM, and MAL) mirror those adopted by other cytology reporting systems and emulate what is already prevalent clinical practice [12,14]. They provide useful information for appropriate clinical management and follow-up. The ROM for each has been estimated by a number of publications [2,15]. One must keep in mind, however, that since ROMs can vary between individual practices, ideally, each laboratory would perform its own assessment, reevaluating these values as needed. ROM values are expected to be refined in the future as more studies based on TIS are performed and published.

A summary description of each category can be found below.

### 2.1. Non-Diagnostic (ND)

Samples providing no useful diagnostic information, such as those with insufficient cellular elements, should be classified in this category. It is a diagnosis of last resort and should only be used after an adequate and representative amount of fluid has been processed and examined. Studies suggest that a minimum of between 50 and 75 mL of fluid should be processed in order to diminish potential false negatives and optimize the test sensitivity [16–21]. If after all efforts the examined slides lack any findings that would be diagnostic or raise the suspicion of a specific diagnosis, they may be classified as ND. The reported ROMs for this category vary between 0 and 100%, with a mean ROM of 17.4% ( $\pm 8.9\%$ ) [2]. The incidence rate of this diagnostic category should vary between 0.2 and 1% [2,15].

Samples reported as ND are non-contributory to clinical care decisions, and a new specimen should be submitted for cytological evaluation, if appropriate, once the effusion reaccumulates.

### 2.2. Negative For Malignancy (NFM)

Samples meeting adequacy criteria and which lack any and all cellular changes characteristic of mesothelial or non-mesothelial malignancy should be classified as NFM [13]. This includes all diseases which lead to the development of benign serous effusions [11]. The reported ROM values for this category vary between 0 and 80% with a mean ROM of 21% ( $\pm 0.3\%$ ) [2,15]. The incidence rate of this diagnosis varies between 70 and 80% [2,15].

In these cases, follow-up and a close correlation with clinical data and imaging studies are adequate clinical management strategies.

### 2.3. Atypia of Undetermined Significance (AUS)

Samples meeting adequacy criteria and that exhibit atypical morphologic features that closely approximate benign, reactive or degenerative changes, but that do not allow for the definitive exclusion of malignancy, should be classified as AUS [13]. The expectation of

finding malignancy in these samples is low, and an AUS diagnosis may be triggered by the detection of benign populations of macrophages or mesothelial cells with reactive or degenerative changes, but also from malignant cells, such as those from low-grade carcinomas or lymphomas. The reported ROM for this category varies between 13 and 100% with a mean ROM of 66% ( $\pm 10.6\%$ ) [2,15]. The expected incidence for this is between 0.6–1.6% [15]. AUS may be used as a place-holder category in a two-step reporting approach, pending ancillary tests [12]. If AUS is the final diagnosis, correlation with clinical and radiological data is advised, as well as repeat sampling, if clinically relevant.

#### 2.4. Suspicious for Malignancy (SFM)

Samples meeting adequacy criteria and showing cytological features usually found in malignant lesions, but that are insufficient in quantity to make a definitive diagnosis of malignancy, should be classified as SFM. This includes samples that raise the suspicion for epithelial, mesothelial, lymphoid, or mesenchymal neoplasms. The reported ROM for SFM varies between 0 and 100% with a mean ROM of 82% ( $\pm 4.8\%$ ) [2,15]. The expected incidence of this category is between 2 and 6.3%, depending on the institutional case-mix [15]. As with AUS, SFM can be thought of as a placeholder category, awaiting the results from ancillary techniques. Clinicians usually regard an SFM diagnosis the same way as they do MAL, taking clinical data into account. As such, SFM should only be used for cases where malignancy is considered highly likely [22].

#### 2.5. Malignant (MAL)—Primary and Secondary

Samples meeting adequacy criteria and showing cytomorphological features that, either alone or in combination with ancillary studies, are diagnostic of a primary or secondary malignancy should be classified as MAL [13]. The reported ROM for MAL varies between 87 and 100% with a mean ROM of 99% ( $\pm 0.1\%$ ) [2,15]. The incidence of this category may be as high as 30% in peritoneal and pleural effusions and higher than 50% in pericardial effusions [15]. A cytological diagnosis of malignancy in effusions is usually treated as definitive and actionable by clinicians and as such, should be as accurate as possible in identifying the type of neoplasm through the use of morphology and ancillary techniques [13].

### 3. Molecular Techniques

Effusions are frequently the first manifestation of malignancy, and thus a first diagnosis is often made based on these cytological specimens [14]. Effusion samples usually contain a high number of viable and well-preserved cells in suspension and thus are adequate for both immunohistochemical and molecular testing [23]. Furthermore, additional material may be found for this latter purpose in the form of supernatants, which contain DNA, RNA, microRNA, and proteins [4,24]. In the context of the TIS, ancillary testing is particularly useful for samples meeting morphologic criteria for the AUS, SFM, and MAL categories. For diagnostic purposes, these tests may be employed to distinguish between a primary and secondary neoplasm, to confirm a diagnosis of malignant mesothelioma (vs. reactive mesothelial hyperplasia), and to correctly classify and determine the primary location of a metastasis. Theranostic molecular tests may also be used for these samples to evaluate potential therapeutic targets [1,25].

#### 3.1. Diagnostic Markers

##### 3.1.1. Mesothelial Proliferations

Malignant mesotheliomas (MM) are the most common primary tumor arising from serous membranes, normally in the context of asbestos exposure [26]. In effusion specimens, MM may show significant morphological overlap with reactive mesothelial hyperplasia and adenocarcinomas from several locations, making this a challenging diagnosis based on morphology alone [27,28].

Immunohistochemistry may be helpful in confirming the nature of malignant cells. CEA, BerEp4, and Claudin-4 are epithelial markers that do not stain mesothelial cells. Conversely, WT1, D2-40, and Calretinin preferentially stain mesothelial cells. Ideally, two of these markers, one of each type, should be employed in the same sample to establish histogenesis. Claudin-4 and Calretinin are the most specific markers for epithelial and mesothelial cells, respectively, and should be preferred for this purpose if available. Loss of BAP-1 is one of the most specific marker of mesothelioma; however, first the mesothelial origin of the cell (with calretinin, for example) should be demonstrated since some non-mesothelial tumors may also shows loss of BAP-1 [14].

Atypical cells of mesothelial origin may raise the differential diagnosis between MM and benign, reactive, mesothelial proliferations. Historically, this has been considered a particularly difficult diagnosis [29]. However, recent developments have determined three markers of particular usefulness: BAP-1 and MTAP immunohistochemistry and CDKN2A fluorescent in situ hybridization (FISH).

BAP-1, which stands for BRCA1-associated protein 1, is a gene is located at the 3p21.1 band and is a transcriptional repressor involved in the long-term silencing of several regulatory genes [30]. Loss of BAP-1 nuclear expression has been shown to be nearly 100% specific for MM [31–34]. Sensitivity may be limited, however. Most sarcomatous mesotheliomas do not show a loss of BAP1, but they also seldom exfoliate to serous cavities. More importantly, up to 40% of epithelial MM, which do exfoliate, may lack BAP1 loss [25,34]. One also must keep in mind that other malignancies, such as malignant melanomas and renal cell carcinomas, may show BAP-1 loss. Thus, the mesothelial origin of the atypical/suspicious cells should be determined before using this marker [14].

MTAP, which stands for methylthioadenosine phosphorylase, an enzyme involved in purine metabolism, is a viable immunohistochemical surrogate marker for the CDKN2A deletion, which is commonly present in MM. MTAP loss of expression is 100% specific for MM and when used in conjunction with BAP-1, can have a sensitivity of up to 90% for epithelial MM arising in the pleural cavity [35–37].

The usefulness of MTAP immunohistochemistry comes from the proximity of its coding gene to the CDKN2A gene locus. The CDKN2A gene is located on the 9p21 band and encodes the p14 and p16 tumor suppressor proteins. Its homozygous deletion is present in around 70% of epithelial pleural MM and 50% of epithelial peritoneal MM. It may be detected by fluorescent in-situ hybridization, reliably differentiating MM and benign reactive mesothelium in effusion specimens, with a specificity of 100%. Unlike MTAP, p14 and p16 immunohistochemistry are not useful markers for CDKN2A deletions in this context [38–43].

Given the high sensitivities but varying specificity of the above methods, at least two should be used in combination [35,44–47].

Interestingly, a recent publication has proposed a combination of only Claudin-4 and BAP-1 as a “2-hit panel” that could at the same time aid in the establishing of histogenesis (epithelial vs. mesothelial) and of benign vs. malignant mesothelial cells. This is because Claudin-4 positivity by itself virtually excludes all benign and malignant mesothelial proliferations, and almost no carcinomas show a loss of BAP1. Additional ancillary tests may then be employed as necessary. Further studies are expected to validate this promising approach [14,48].

### 3.1.2. Metastatic Neoplasms

Metastatic neoplasms to serous cavities most often include carcinomas from the breast and lung, but malignant melanomas, soft tissue sarcomas, and other malignancies may also involve these cavities [49,50].

Once a malignant population of cells has been established as epithelial in nature, as per the method defined above, one should proceed in characterizing it to the full extent the material will allow. In addition to looking for morphological clues, the same markers that are used in tissue sections to determine the differentiation and primary origin of

carcinomas may be used, such as CK7 and 20, GATA-3 for tumors from the breast and urothelium, PAX8 for tumors from the gynecological tract, thyroid, kidney, and thymus, CDX2 for tumors with intestinal differentiation, mostly from the gastrointestinal tract, TTF-1 for tumors from the lung and thyroid, and PSA, ERG, and NKX3.1 for tumors from the prostate [14,51,52].

When using these markers, one must keep in mind that specificity is never 100%. Particularly, GATA-3 may stain a significant percentage of malignant mesotheliomas, and as such, an epithelial differentiation must always be securely established before giving significance to this or other markers [53].

If melanoma is suspected, the presence of pigment may give provide a clue to a proper diagnosis. Negativity for keratins and positivity for S100, HMB-45, MITF, and Melan A usually enable a definitive diagnosis [54].

Sarcomas may pose an additional challenge, due to their rarity, but also due to occasional cross positivity with keratins. Claudin-4 may help in this regard, since it appears to be more specific for truly epithelial neoplasms. In the correct clinical context, and if there is enough material, immunohistochemical markers such as CD10, CD31, CD34, CD99, D2-40, Desmin, SMA, MyoD1, MDM1, and S100 may aid in making a tentative or definitive diagnosis [55–57]. FISH and other molecular tests may also be used to test for entity defining translocations, further aiding in this diagnostic process [58].

### 3.1.3. Lymphoproliferative Disorders

Lymphoid neoplasms frequently involve serous cavities [59]. Lymphocytosis, comprised of small lymphocytes, are almost always benign and secondary to infectious processes, but when present in large numbers, these may raise concern and lead to a specimen being classified as AUS according to the TIS. When large, atypical, lymphocytes are numerous, a sample should be classified as SFM or MAL, but phenotyping may still be necessary to correctly classify the lymphoma and reach an accurate diagnosis. In these cases, a cell-block may be performed, and immunohistochemistry and Epstein-Barr virus in situ hybridization (EBER-ISH) may be used as one would on tissue samples. If a sample is obtained fresh and there is a high clinical suspicion for lymphoma, flow cytometry may successfully be used to provide an accurate phenotypic analysis [14]. Polymerase chain reaction, FISH, and cytogenetics have also been shown in the literature to be useful for the diagnosis of hematolymphoid neoplasms [14].

A summary of these ancillary diagnostic tests can be found in Table 1.

## 3.2. Theranostic Markers

The repertoire of theranostic markers is expanding every day, as more is known about tumor biology and more targets for personalized therapy are identified. Currently, theranostic markers play an important role in the management of lung and breast cancers. As was previously established, effusion samples provide ample and adequate material for molecular testing. In the context of an MAL diagnosis, effusion samples may be used for this purpose [14].

### 3.2.1. Lung Cancer

In the context of non-small cell lung cancer (NSCLC), the testing of several genes is recommended, particularly of EGFR (epithelial growth factor receptor), ALK (anaplastic lymphoma kinase), and ROS1. Testing for these three genes is recommended by current guidelines in advanced lung adenocarcinomas, along with BRAF [14,60,61]. NTRK (neurotrophic tyrosine kinase receptor) 1/2/3 inhibitors have also been approved for use in both adult and pediatric patients with advanced solid tumors, including lung adenocarcinoma, and an increasing number of centers is testing for NTRK translocations in their routine practice [14,62,63]. Immunohistochemical markers have been developed for ALK, ROS1, BRAF, and the NTRK family. ALK and ROS1 immunohistochemistry have been validated for use in effusion cytology samples, and based on previous research, it is reasonable to

expect that most immunohistochemistry markers should provide interpretable results, particularly if used in cell-blocks [64]. However, molecular testing in the form of FISH or next-generation sequencing (NGS) is still required. NGS is considered of particular relevance and the method of choice, as it enables multiplexing, that is, sequencing of multiple genes using a single sample, and is recommended over alternative techniques in identifying treatment options in the context of lung cancer, particularly when testing beyond EGFR, ALK, and ROS1 and/or if sample material is scarce [65–68].

**Table 1.** Ancillary tests that may be used in the context of AUS, SFM or MAL for diagnostic purposes in serous effusion samples\*.

Diagnosis	Ancillary Tests
Reactive mesothelial cells vs. Carcinomas	Epithelial markers—IHC: Claudin-4 BerEP4 CEA Mesothelial markers—IHC: Calretinin WT1 D2-40
Reactive mesothelial cells vs. Malignant Mesothelioma	“Brescia Panel”—HC: Claudin-4 (epithelial) + BAP-1 (Claudin-4 stains almost all carcinomas; BAP-1 is almost never lost in carcinomas, lost in malignant mesothelioma) At least two used in conjunction—most useful for epithelioid MMs IHC: BAP1, MTAP FISH: CDKN2A
Hematolymphoid neoplasms	IHC: CD3, CD5, CD20, CD21, CD23, CD 45, CD5, CD10, Bcl-2, Bcl-6, MUM1, Ki-67, K and λ light chain etc. ISH: EBER, K and λ light chain FISH: ALK, MYC, BCL-2, BCL-6, CCND1, etc.
Epithelial neoplasms	IHC: CK7, CK20, PAX-8 (kidney/gyn tract), GATA-3 (urothelium, breast), TTF-1 (lung, thyroid), PSA, ERG and NKX3.1 (prostate), etc.
Malignant melanoma	IHC: S100, Melan-A, HMB-45, MITF
Soft tissue sarcomas	IHC: CD10 (endometrial stromal neoplasms), CD31, CD34, D2-40 (vascular sarcomas), CD99 (Ewing, synovial sarcomas, others), Desmin, SMA (leiomyosarcomas), MyoD1 (rhabdomyosarcomas), MDM1 (liposarcomas), S100 (malignant peripheral nerve sheath tumors, clear cell sarcoma, others), etc. FISH/Molecular tests: for specific translocations

\* All sample types may be used as long as sufficient cellularity is available, as in tissue. For IHC, cell-blocks are preferred.

Another molecule of interest in the context of lung cancer is PD-L1 (programmed death-ligand 1). Testing of PD-L1 expression by immunohistochemistry is current routine practice as several inhibitors of this pathway have been approved for clinical use and even more are in the process of undergoing clinical trials [69].

PD-L1 immunohistochemistry has been well validated for use in serous effusion samples, particularly on cell-block material. One should be wary of false positive staining in macrophages. Evaluation of PD-L1 expression may be difficult in inflammatory cells [14].

### 3.2.2. Breast and Ovarian Cancers and Other Malignancies

In the context of breast cancer, testing for HER2, estrogen and progesterone receptors, and Ki67 is mandatory. In the context of ovarian cancer, hormonal receptor testing is useful in defining therapeutic strategies, as well as testing for BRCA1/2 mutations. In colon cancer and other malignancies, such as carcinomas of the endometrium, testing

for mismatch repair (MMR) proteins is increasingly important, both to select patients for immunotherapy as well as screening for Lynch syndrome [70].

Immunohistochemistry and FISH for HER2 testing have been validated for use in effusion samples. The same is true for hormonal receptors and MMR proteins. Molecular testing for BRCA1/2 mutations may be performed in these samples as well, preferably through simple sequencing or NGS [14,70].

For further details on the use of theranostic tests on serous effusion samples, see Table 2.

**Table 2.** Ancillary tests for use in theranostic purposes in the most common neoplasms involving serous cavities \*.

Diagnosis	Genes	IHC	Molecular Tests
NSCLC	EGFR	No	Simple sequencing, RT-PCR, NGS (DNA)
	ALK1	Yes	FISH, NGS (DNA, RNA)
	ROS1	Yes Confirmatory molecular test required	FISH, NGS (DNA, RNA)
	BRAF	Yes For V600E mutation	Simple sequencing, RT-PCR, NGS (DNA)
	TRK family	Yes Confirmatory molecular test required	FISH, NGS (DNA, RNA)
	KRAS, NRAS, BRAF, RET, MET, PIK3CA, etc.	No	NGS (DNA, RNA) Preferred, as it enables multiplexing, including genes above
	PD-L1	Yes	No
Breast and ovarian cancer	Hormonal receptors (ER, PR)	Yes	No No
	HER2	Yes May require confirmation by FISH	FISH
	KI67	Yes	No
	BRCA1/2	No	Simple sequencing, NGS (DNA)
Other neoplasms (colon, endometrium, etc.)	MMR	Yes	RT-PCR (for microsatellite instability), direct sequencing of MMR proteins
	Other genes	Varies, mostly no	Varies, NGS is preferred

\* All sample types may be used as long as sufficient cellularity is available, as in tissue. For IHC, cell-blocks are preferred.

#### 4. Discussion

TIS applies to all serous fluid cytology samples. It is a step forward in the standardization of effusion cytology diagnosis and should be particularly useful in minimizing the use of “uncertain” diagnoses, including AUS and SFM categories.

These indeterminate categories are presented as an option of last resort. As we have shown, when faced with atypical cellular populations fitting the AUS and SFM categories, cytopathologists now have at their hands, immunohistochemical and molecular tests, validated for this type of sample and of similar efficacy for tissue, with a few caveats.

Using these methods, in the context of malignancy, cytology reports can aim to be as definitive as possible. A final diagnosis of malignant mesotheliomas should be rendered when both morphological and molecular criteria support the diagnosis. Secondary malignancies should be classified to the greatest extent possible, and this includes phenotyping atypical lymphoid populations through immunohistochemistry or flow cytometry, as well as the identification of the primary site and histological type (if possible) of metastatic epithelial tumors.

As TIS is implemented, retrospective and prospective studies should provide more information about the management of the different categories and lead to refinements of the classification system. As it stands, the authors expect it should help cytopathologists better manage samples and also improve management strategies for patients at the clinical level.

The importance of serous effusion cytology is bound to increase in the future in the context of theranostics, as these samples are easy to obtain and contain abundant material for molecular testing. Pathologists will keep playing a central role in guiding this process, by determining adequacy, selecting appropriate ancillary tests for the clinical context, and interpreting immunohistochemistry, when appropriate.

We believe this will be an area of active research in the near future as new therapeutic targets are discovered and new drugs enter the clinical practice.

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