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Current clinical and research fluid biomarkers to aid risk stratification of pancreatic cystic lesions

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ABSTRACT
Pancreatic cystic lesions (PCL) are composed of a heterogeneous group of entities that are increasingly diagnosed, generally as incidental findings in asymptomatic patients. In conjunction with this growing incidence, the potential for malignant transformation of mucin-producing cysts makes PCL a challenging clinical conundrum for the clinician, patient, and healthcare system. Cyst characterization based on morphology is often difficult and inaccurate. Therefore, several intracystic fluid biomarkers have been evaluated as ancillary testing to enhance the difficult balance between sparing a patient from an unnecessary high-risk pancreatic surgery and missing the opportunity
to prevent or diagnose pancreatic adenocarcinoma at an early disease stage. There are two questions that are key to guide the care of patients with PCL: 1) is it a non-mucinous cyst that does not require any follow-up? and 2) if mucinous, does the cyst harbor advanced neoplasia (high-grade dysplasia or invasive carcinoma) that requires surgical resection, or is it a low-risk lesion that will benefit from a surveillance program? The purpose of this review is to give a general and practical overview of the different cyst fluid biomarkers that have been studied to address these specific questions, from classic biochemical markers such as carcinoembryonic antigen to novel genetic and epigenetic markers such as microRNA or intracystic bacterial DNA.

**Keywords:** Biomarkers. Cyst fluid. Genetic markers. Pancreatic cyst. Pancreatic intraductal neoplasms.

**INTRODUCTION**

Pancreatic cystic lesions (PCL) are increasingly detected due to the widespread use of high-resolution, cross-sectional abdominal imaging. The prevalence varies with the technique (higher in studies using magnetic resonance imaging) and the population considered (increases with age), with an estimated prevalence of 8 % (95 % confidence interval [CI]: 4-14 %) (1). PCL include a wide range of entities (Table 1). Aside from solid pancreatic tumors with cystic degeneration, which are rare and generally identified by cytopathologic techniques, cystic neoplasms may be further subcategorized as non-mucin or mucin-producing, the latter representing approximately 60 % of PCL (1). Intraductal papillary mucinous neoplasm (IPMN), particularly side-branch IPMN, is the most common type of PCL. Accurate identification of mucinous cysts (IPMN and mucinous cystic neoplasm [MCN]) is crucial as they are macroscopic precursors to pancreatic adenocarcinoma (PDAC), progressing from low-grade dysplasia (LGD) to high-grade dysplasia (HGD) to invasive carcinoma (2). If a mucinous cyst is confirmed, the ideal approach would be to remove it due to its malignant potential, as with colon adenomas. Even though less invasive endoscopy-guided therapeutic approaches are
being developed as an alternative to surgery, resection of PCL is the only currently approved treatment. Nevertheless, due to the high morbidity (30 %), mortality (2 %), and long-term complications related to pancreatic surgery for PCL, resection is only justified in patients with more advanced disease, which include mucinous cysts harboring HGD or invasive carcinoma (2,3). These two disease stages (HGD and invasive carcinoma) can be unified under the term “advanced neoplasia”. Furthermore, malignant transformation only occurs in a small number of low-risk lesions, and the progression rate is relatively slow (8 % at ten years) (4). Therefore, in the absence of symptoms, a cyst with LGD can be monitored if the patient is a surgical candidate (2). In contrast, non-mucinous lesions do not require any follow-up, similar to the clinical scenario of hyperplastic rectal polyps.

When considering the high number of asymptomatic patients with incidental PCL and the globally low risk of malignant transformation of mucinous cysts, what could be a window of opportunity to diagnose PDAC at an early, curable stage has become a real dilemma for clinicians, an understandable concern to patients, and an increasing burden to any healthcare system. The morbidity associated with pancreatic surgery makes it equally critical to avoid a missed cancer and overtreatment. Current risk prediction models based on morphologic findings have a low specificity to identify those few patients with mucinous PCL and advanced neoplasia that benefit from surgery. Even if worrisome or high-risk features are present, 17 % of cysts that undergo resection have no malignant potential, and the majority of resected mucinous lesions do not harbor advanced neoplasia (5). In addition, the high specificity of preoperative cytology in cystic fluid via endoscopic ultrasound (EUS) is hampered by its suboptimal sensitivity (57 %) due to scant fluid cellularity (6).

From a practical point of view, PCL can be divided into three groups for their clinical management: non-mucinous PCL that have no malignant potential and do not require surveillance, mucinous PCL without advanced neoplasia that will benefit from monitoring, and mucinous PCL with advanced neoplasia that require surgery. In this clinical scenario, fluid biomarkers have evolved in an attempt to assist us in “finding the needle in the haystack”. We aim to provide an overview of the more relevant biomarkers studied in cyst fluid to discriminate and stratify PCL into these three groups.
(Table 2). Of note, fluid sampling, which can be easily obtained during EUS (Fig. 1), should only be performed if the results are expected to change the patient’s clinical management: 1) if cyst type cannot be characterized by morphology and cyst size is > 15 mm; or 2) if there are high-risk features suggestive of advanced neoplasia, which vary among the different guidelines but generally imply a cyst size > 30 mm, main duct dilation, mural nodule, thickened cyst wall, or interval cyst growth (2).

BIOCHEMICAL MARKERS

Available for clinical use

Carcinoembryonic antigen (CEA)
CEA was one of the first cyst fluid biomarkers studied and is the most widespread in clinical practice. Almost two decades ago, a cutoff for CEA of > 192 ng/mL was shown to differentiate mucinous from non-mucinous lesions (7). A recent meta-analysis including 31 studies confirmed a high specificity (80 %; 95 % CI: 76-83 %) but a low sensitivity (67 %; 95 % CI: 65-70 %) (8). Raising the threshold (> 800 ng/mL) further increases the specificity for mucinous lesions, while lowering the cutoff (< 5 ng/mL) significantly improves specificity for non-mucinous cysts but at the cost of further decreasing sensitivity (9). Even if flawed, CEA (which requires 0.5-1 mL of fluid) is recommended by current guidelines as the first analysis to distinguish mucinous from non-mucinous PCL (2). However, CEA does not correlate with the degree of dysplasia (6).

Glucose
Low intracystic glucose (< 50 mg/dL) has been recently associated with mucin-producing cysts with a sensitivity of 91 % (95 % CI: 86-94 %) and specificity of 75 % (95 % CI: 68-82 %) (8). Interestingly, intracystic glucose levels do not correlate with cyst size or blood glucose levels, and are not influenced if the patient is diabetic (10). However, the mechanisms behind the reduction of intracystic glucose in mucinous cysts remain unexplained. Onsite intracystic glucose analysis using a standard
A glucometer or a reagent strip is feasible and shows good correlation with laboratory glucose, providing an immediate diagnosis with only 2 μL of fluid (11). Nevertheless, other studies have reported reading errors of the glucometer due to high viscosity (12). Intracystic glucose seems an attractive biomarker to identifying mucinous cysts as it may outperform CEA in terms of sensitivity; it is affordable, reproducible, widely available, and requires 50 μL of fluid (even less with a glucometer). However, the number of studied patients thus far is small and requires future validation. Furthermore, the combination of both glucose and CEA probably provides more accurate information (10). Unfortunately, the degree of dysplasia cannot be accurately determined by intracystic glucose levels.

**Amylase**

Higher levels of amylase, an enzyme secreted by acinar cells, are found in pseudocysts but are not specific. In contrast, a cutoff < 250 U/L practically excludes a pseudocyst (specificity 98%) but does not differentiate non-mucinous from mucinous cysts (9). Furthermore, as IPMN are connected to the ductal system and MCN are not, higher levels could be expected in IPMN. However, intracystic amylase levels do not distinguish a MCN from an IPMN (9).

**Carbohydrate antigen 19.9 (CA 19.9)**

CA 19.9 is a well-known serum marker for PDAC, and several studies have evaluated its role in cyst fluid to differentiate mucinous from non-mucinous cysts and degree of dysplasia. A recent meta-analysis found no significant differences between mucinous (mean value 35,595 U/mL) and non-mucinous (1,004 U/mL) cysts, with a pooled sensitivity of 68% and a specificity of 68% (13). Hence, any cyst can present with an elevation of fluid CA 19-9 substantially above the serum reference value (37 U/mL). Other tumor markers investigated include CA 125, CA 72-4, and CA 15-3, but they do not discriminate between cyst subtypes (7).

**Research fluid biomarkers**
**Interleukin-1 beta (IL-1β)**

Several intracystic cytokines have been investigated with the hypothesis that dysplasia may induce a pro-inflammatory microenvironment. In a small study, IL-1β, a cytokine secreted into the extracellular space, showed higher fluid levels in IPMN with HGD or invasive carcinoma, and discriminated them from low-risk IPMN regardless of type (main or branch). A cutoff value of 1.26 pg/mL showed a specificity of 95% and a sensitivity of 79% (14). Recent studies have confirmed higher intracystic IL-1β in IPMN with advanced neoplasia (15,16).

**Mucins (MUC)**

Pancreatic mucins are glycosylated proteins involved in the lubrication of the epithelial duct lining, and have been implicated in carcinogenesis (17). Extracellular mucin can be assessed by mucicarmine or Alcian blue but is only observed in 60% of mucinous cysts, mainly due to inadequate cytological samples for staining (18). Cystic fluid MUC2 and MUC4 levels were higher in lesions harboring advanced neoplasia in a small study, with a high sensitivity and specificity (92% and 94%, respectively) (17).

**Prostaglandin E2 (PGE2)**

Cyclooxygenase-2 has an important role in inflammation and carcinogenesis, and is overexpressed in the epithelium of mucinous PCL compared to non-mucinous cysts (19). The expression of its enzymatic product PGE2 has been explored in cyst fluid to assess the degree of dysplasia, showing that IPMN with HGD or invasive cancer display higher levels than those with LGD. Combined with CEA > 192 ng/mL, PGE2 > 0.5 pg/µL demonstrated a high accuracy (86%) for detecting advanced neoplasia, regardless of the use of non-steroidal anti-inflammatory medications (20). In a recent study, the signature IL-1β, MUC2, and PGE2 accurately discriminated IPMN from HGD (16).

**Vascular endothelial growth factor-A (VEGF-A)**

VEGF-A is involved in blood-cell growth, and high intracystic levels have been shown in serous cystic neoplasms, probably due to the rich vascularization of these lesions. A recent study confirmed that elevated VEGF-A (> 5,000 pg/mL) is highly specific (98%)
for serous cystic neoplasms (although with a low sensitivity), and may be useful to avoid unnecessary surgery or follow-up (5).

GENETIC AND EPIGENETIC BIOMARKERS
Advances in technology, particularly next-generation sequencing (NGS), offering enhanced sensitivity at reduced costs, are enabling a rapid progress in biomarker discovery in the field of PCL. These novel biomarkers may also overcome the limitation of the amount of fluid, as they require low fluid volumes (0.2 mL) (5).

Available for clinical use

Pathogenic genomic variants
DNA from denudated epithelial lining cells can be analyzed in cyst fluid for pathogenic genomic variants.

— KRAS/GNAS. Activating mutations in oncogene KRAS, found in nearly all PDAC, have shown promise to identify mucin-producing cysts. In fact, KRAS mutations are present in both IPMN (76 %) and MCN (35 %) (5). In a meta-analysis of 731 patients, the sensitivity and specificity for mucinous differentiation were 46 % (95 % CI: 42-51 %) and 97 % (95 % CI: 92-99 %), respectively (21). However, KRAS can occur in non-mucinous cysts and does not reliably characterize the degree of dysplasia (5,6,21). Regarding GNAS, while IPMN often harbor mutations in GNAS (56 %) regardless of the grade of dysplasia, MCN usually do not have mutations in this oncogene (5). Mutations in either KRAS or GNAS have been reported in 86 % of IPMN, and a mutation in both genes in 47 % (5). Therefore, fluid KRAS mutation suggests — but does not exclude — a mucinous cyst, and a GNAS mutation, although less frequent, is highly specific (96 %) for mucinous lesions and particularly indicative of IPMN (5,22). Due to its low sensitivity, KRAS/GNAS mutational analysis alone is not optimal for screening for mucinous cysts as they seem to occur early in the neoplastic transformation process and do not discriminate advanced neoplasia that may benefit from surgical resection (6). Even so, in certain situations, KRAS/GNAS assessment can be of use, as they may
reclassify cysts that remain indeterminate after imaging, CEA, and cytology into mucinous lesions (2).

— VHL. Mutations of this tumor suppressor gene are observed in 43 % of serous cystadenoma and almost absent in all other types of PCL, as recently confirmed in a large study (5). Therefore, it may be useful to confirm a serous cystadenoma. Fluid DNA panels have been evaluated to identify pathogenic genomic variants associated with advanced neoplasia. In this sense, mutations/deletions in PIK3CA, PTEN, TP53, CDKN2A, and SMAD4 in mucin-producing cysts have been associated with advanced neoplasia (5,22). However, although DNA analysis seems to be useful to characterize the type of cyst, twelve years after the PANDA study, the first large study evaluating cyst fluid DNA, we are still digging to find those variants that reliably identify advanced neoplasia.

**Research fluid biomarkers**

**Telomerase**

As telomerase activity is often activated in malignant cells, its fluid levels have been evaluated to assess the degree of dysplasia. High telomerase activity (\( \geq 730 \text{ copies/\muL} \)) identified advanced neoplasia with a specificity of 93 % in a single-center study. Specifically, cyst fluid telomerase activity had a high performance in cysts with morphological worrisome features (23).

**Epigenetic alterations**

Mechanisms leading to changes in gene expression that do not involve a permanent change in the DNA sequence have also been explored in pancreatic cyst fluid.

— DNA methylation. Several genes have been identified that undergo aberrant methylation during pancreatic carcinogenesis. Therefore, methylated DNA markers have been interrogated in cystic fluid and found to discriminate between HGD or invasive carcinoma and LGD in IPMN. Specifically, two markers (TBX15, BMP3) have been validated, showing a sensitivity and specificity above 80 % (24).
— **MicroRNA.** MicroRNAs are non-coding RNAs that regulate gene expression at the post-transcriptional level. They have an increasingly recognized role in early carcinogenesis and show great potential as diagnostic or prognostic biomarkers in several diseases. A panel of 9 microRNAs (miR18a, miR24, miR30a-3p, miR92a, miR99b, miR106b, miR142-3p, miR342-3p, and miR532-3p) have been identified in cyst fluid and validated by a different group, confirming a high specificity (100 %) for advanced neoplasia identification but a very low sensitivity (10 %) (25). However, the role of cyst fluid microRNA remains to be defined.

**Microbiome**

An inherent pancreatic microbiome is gaining increasing relevance as a theranostic tool in PDAC. As the microbiome may promote oncogenesis through local immune modulation, its role in identifying advanced neoplasia in mucinous cysts has been explored. In a recent study, IPMN harboring HGD or invasive cancer had significantly higher intracystic bacterial DNA copies compared to LGD, suggesting that bacteria could promote progression from LGD to HGD (15). At the genus level, HGD lesions were enriched in *Granulicatella, Serratia* and *Fusobacterium. Fusobacterium nucleatum*, a well-recognized oncobacterium, was predominantly seen in the cyst fluid of HGD IPMN.

**COMBINED TESTING**

A comprehensive test including clinical characteristics, morphological features, and cyst fluid biochemical and genetic markers has been recently identified in a large multicenter study. The results hold promise to classify patients into those that require optimally timed surgical intervention (mucinous cysts with advanced neoplasia), participation in a surveillance program (low-risk mucinous cysts), or no need for further routine surveillance (non-mucinous cysts) (5).

**CONCLUSIONS**

PCL management is clinically challenging as the majority of cysts will not become malignant and the rate of malignant transformation is low and difficult to predict.
Several types of biomarkers have been investigated to differentiate PCL without malignant potential that do not require any subsequent follow-up from those with advanced neoplasia requiring surgery. An important limitation is that most published studies use surgical pathology as the criterion standard to assess the diagnostic accuracy of fluid biomarkers. As surgical cohorts comprise a higher proportion of mucinous and advanced neoplasia cysts, the results and interpretation may not be generalizable to all PCL patients. Accordingly, the performance of the biomarkers is lower when applied preoperatively, as the sensitivity and specificity of a particular test will vary with disease prevalence.

As biomarker levels overlap between cysts and no test is perfect, risk stratification strategies that synergistically combine cyst morphology and fluid biomarkers will be the most likely and reasonable way to move forward (5). Keeping this in mind, a practical approach considering the biomarkers with more robust evidence is shown in figure 2. A combination of high intracystic CEA and low glucose can reasonably suggest a mucinous PCL, and both markers are widely available and affordable. If an indeterminate and precise diagnosis of a mucinous cyst will change management, mutational analysis of KRAS/GNAS is highly specific for mucin-producing cysts and can be considered when acknowledging its low sensitivity. However, none of them will determine the degree of cystic epithelial dysplasia. Although promising, efforts to identify advanced neoplasia by novel biomarkers are in early clinical development, and they will require standardization and adequate prospective independent validation. Furthermore, we are still far removed from the clinical ideal of predicting the likelihood and timing of malignant transformation among low-risk mucinous cysts. Nevertheless, the evolving role of precision medicine through fluid genomic biomarkers will certainly be a key to tailored, personalized PCL management in the not too distant future.
REFERENCES


Table 1. Classification of pancreatic cystic lesions

<table>
<thead>
<tr>
<th>Neoplastic</th>
<th>Cystic neoplasms</th>
<th>Non-mucinous</th>
<th>Mucinous</th>
<th>No malignant potential</th>
<th>Malignant potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serous cystadenoma</td>
<td>MCN</td>
<td>No malignant potential</td>
<td>Malignant potential</td>
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<td></td>
<td></td>
<td></td>
<td>IPMN</td>
<td>Branch</td>
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<td></td>
<td>Main</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Mixed</td>
<td></td>
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<tr>
<td></td>
<td>Cystic degeneration of solid non-mucinous tumors</td>
<td>Solid pseudopapillary neoplasm</td>
<td>Cystic neuroendocrine tumor</td>
<td>Cystic ductal adenocarcinoma</td>
<td>Cystic acinar-cell carcinoma</td>
</tr>
<tr>
<td></td>
<td>No epithelial lining</td>
<td>Inflammatory fluid collections</td>
<td>Pseudocyst</td>
<td>Parasitic cyst</td>
<td>No malignant potential</td>
</tr>
<tr>
<td></td>
<td>Epithelial lining</td>
<td>Epithelial cyst or “true cyst”</td>
<td>Retention cyst</td>
<td>Lymphoepithelial cyst</td>
<td>Mucinous non-neoplastic cyst</td>
</tr>
</tbody>
</table>

Modified from (2). MCN: mucinous cystic neoplasm; IPMN: intraductal papillary mucinous neoplasm. The most frequent pancreatic cystic lesions are shown in italics (cystic neoplasms and inflammatory fluid collections occurring in the setting of acute or chronic pancreatitis).
Table 2. Fluid biomarkers studied in pancreatic cystic lesions

<table>
<thead>
<tr>
<th>Fluid biomarkers studied in PCL</th>
<th>GENETIC AND EPIGENETIC BIOMARKERS</th>
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<tbody>
<tr>
<td>BIOCHEMICAL BIOMARKERS</td>
<td>DNA pathogenic genomic variants</td>
</tr>
<tr>
<td>CEA</td>
<td>Telomerase activity</td>
</tr>
<tr>
<td>Glucose</td>
<td>Methylated DNA</td>
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<tr>
<td>Amylase</td>
<td>microRNA</td>
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<tr>
<td>CA 19.9</td>
<td>Microbiome</td>
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<tr>
<td>Interleukins (IL-1β)</td>
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<tr>
<td>Mucin</td>
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<td>Prostaglandins (PGE2)</td>
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<td>VEGF-A</td>
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<table>
<thead>
<tr>
<th>Fluid biomarkers suggested to discriminate</th>
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<tbody>
<tr>
<td>1. Type of cyst</td>
<td>Other</td>
</tr>
<tr>
<td>Mucinous</td>
<td></td>
</tr>
<tr>
<td>CEA ↑</td>
<td>Amylase ↓ (against pseudocyst)</td>
</tr>
<tr>
<td>Glucose ↓</td>
<td>VEGF-A ↑ (serous cystadenoma)</td>
</tr>
<tr>
<td>KRAS mutation (IPMN/MCN)</td>
<td>VHL mutation (serous cystadenoma)</td>
</tr>
<tr>
<td>GNAS mutation (IPMN)</td>
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<thead>
<tr>
<th>2. Presence of advanced neoplasia (HGD or invasive carcinoma)</th>
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<tr>
<td>IL-1β ↑</td>
<td>Pathogenic genomic variants (PIK3CA, PTEN, TP53, CDKN2A, SMAD4)</td>
</tr>
<tr>
<td>MUC2, MUC4 ↑</td>
<td>Telomerase activity ↑</td>
</tr>
<tr>
<td>PGE2 ↑</td>
<td>methylated DNA markers</td>
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<tr>
<td></td>
<td>Intracystic microbiome</td>
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<table>
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<tr>
<th>Fluid biomarkers by pancreatic cyst lesion*</th>
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<tbody>
<tr>
<td>IPMN</td>
<td>PSEUDOCYST</td>
</tr>
<tr>
<td>↑ ↓ Amylase</td>
<td>↑ ↑ ↑ Amylase</td>
</tr>
<tr>
<td>↑ ↑ CEA</td>
<td>↓ CEA</td>
</tr>
<tr>
<td>↓ ↓ Glucose</td>
<td></td>
</tr>
<tr>
<td>KRAS mutated</td>
<td></td>
</tr>
<tr>
<td>GNAS mutated</td>
<td></td>
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<tr>
<td>MCN</td>
<td>SEROUS CYSTADENOMA</td>
</tr>
<tr>
<td>↑ ↓ Amylase</td>
<td>↑ ↓ Amylase</td>
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</table>
PCL: pancreatic cystic lesions; IPMN: intraductal papillary mucinous neoplasm; MCN: mucinous cystic neoplasm; HGD: high-grade dysplasia.
Fig. 1. Fluid of a pancreatic cyst collected via endoscopic ultrasound. A. Endosonographic view of the cyst being aspirated. B. Aspirated fluid. A cystic size of 10-15 mm is the minimum required to obtain a satisfactory fluid volume for subsequent analysis. It is generally recommended to completely drain the aspirated cyst.
Fig. 2. Proposal for the practical application of fluid biomarkers in pancreatic cystic lesions (EUS: endoscopic ultrasound; IPMN: intraductal papillary mucinous neoplasm; MCN: mucinous cystic neoplasm; CEA: carcinoembryonic antigen; HGD: high-grade dysplasia. *If fluid analysis will change the patient’s clinical management. †Determined by cytology as no fluid biomarker is currently available to confirm advanced neoplasia).