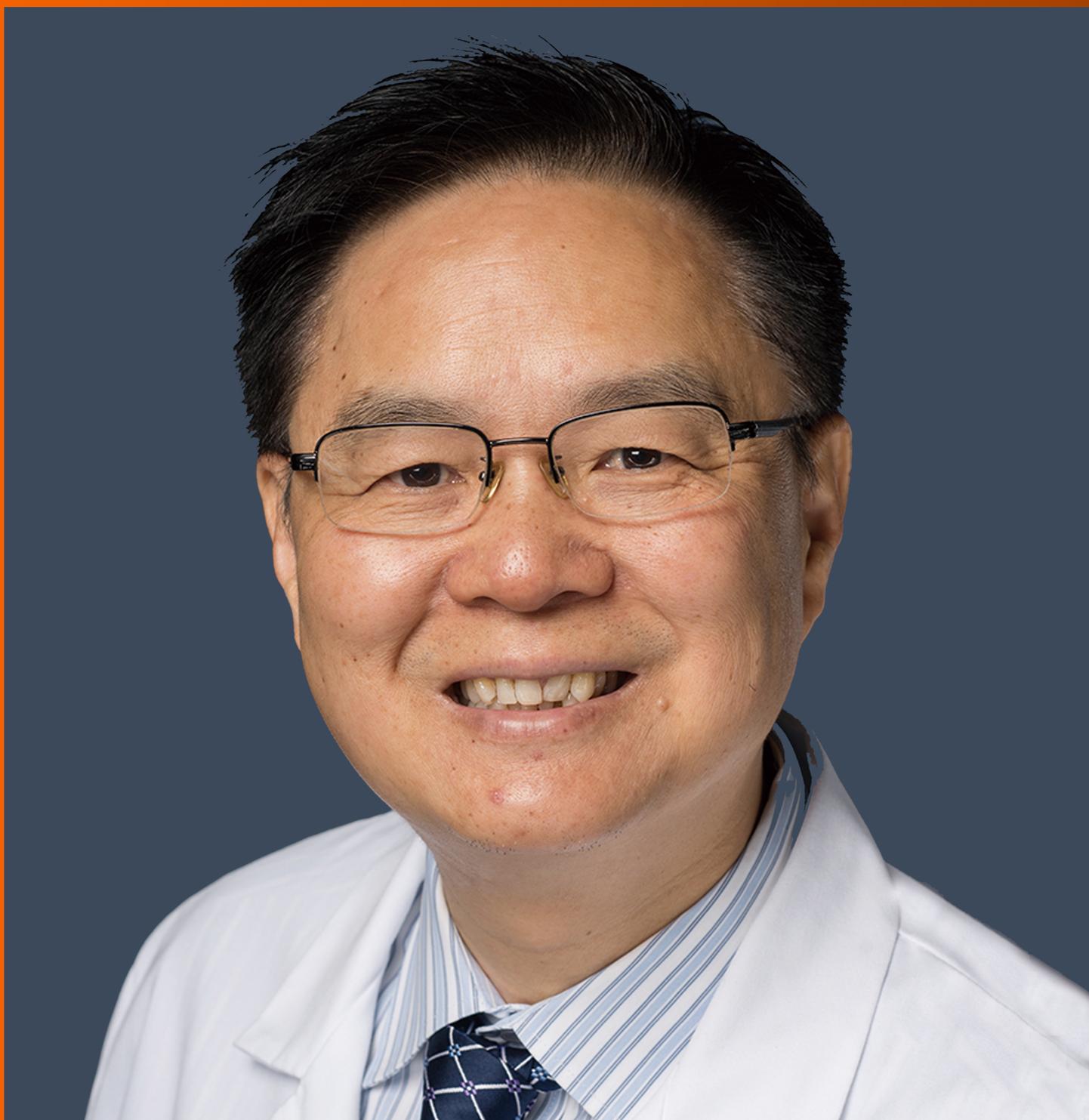


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AIMS AND SCOPE

The primary aim of *World Journal of Gastrointestinal Endoscopy* (WJGE, *World J Gastrointest Endosc*) is to provide scholars and readers from various fields of gastrointestinal endoscopy with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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Observational Study

Molecular analysis of pancreatic cystic neoplasm in routine clinical practice

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Abstract

BACKGROUND

Cystic pancreatic lesions consist of a wide variety of lesions that are becoming increasingly diagnosed with the growing use of imaging techniques. Of these, mucinous cysts are especially relevant due to their risk of malignancy. However, morphological findings are often suboptimal for their differentiation. Endoscopic ultrasound fine-needle aspiration (EUS-FNA) with molecular analysis has been suggested to improve the diagnosis of pancreatic cysts.

AIM

To determine the impact of molecular analysis on the detection of mucinous cysts and malignancy.

METHODS

Informed consent statement:

Informed consent was obtained from all patients prior to study inclusion.

Conflict-of-interest statement:

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An 18-month prospective observational study of consecutive patients with pancreatic cystic lesions and an indication for EUS-FNA following European clinical practice guidelines was conducted. These cysts included those > 15 mm with unclear diagnosis, and a change in follow-up or with concerning features in which results might change clinical management. EUS-FNA with cytological, biochemical and glucose and molecular analyses with next-generation sequencing were performed in 36 pancreatic cysts. The cysts were classified as mucinous and non-mucinous by the combination of morphological, cytological and biochemical analyses when surgery was not performed. Malignancy was defined as cytology positive for malignancy, high-grade dysplasia or invasive carcinoma on surgical specimen, clinical or morphological progression, metastasis or death related to neoplastic complications during the 6-mo follow-up period. Next-generation sequencing results were compared for cyst type and malignancy.

RESULTS

Of the 36 lesions included, 28 (82.4%) were classified as mucinous and 6 (17.6%) as non-mucinous. Furthermore, 5 (13.9%) lesions were classified as malignant. The amount of deoxyribonucleic acid obtained was sufficient for molecular analysis in 25 (69.4%) pancreatic cysts. The amount of intracystic deoxyribonucleic acid was not statistically related to the cyst fluid volume obtained from the lesions. Analysis of *KRAS* and/or *GNAS* showed 83.33% [95% confidence interval (CI): 63.34-100] sensitivity, 60% (95%CI: 7.06-100) specificity, 88.24% (95%CI: 69.98-100) positive predictive value and 50% (95%CI: 1.66-98.34) negative predictive value ($P = 0.086$) for the diagnosis of mucinous cystic lesions. Mutations in *KRAS* and *GNAS* were found in 2/5 (40%) of the lesions classified as non-mucinous, thus recategorizing those lesions as mucinous neoplasms, which would have led to a modification of the follow-up plan in 8% of the cysts in which molecular analysis was successfully performed. All 4 (100%) malignant cysts in which molecular analysis could be performed had mutations in *KRAS* and/or *GNAS*, although they were not related to malignancy ($P > 0.05$). None of the other mutations analyzed could detect mucinous or malignant cysts with statistical significance ($P > 0.05$).

CONCLUSION

Molecular analysis can improve the classification of pancreatic cysts as mucinous or non-mucinous. Mutations were not able to detect malignant lesions.

Key Words: Pancreatic cysts; Molecular analysis; Next-generation sequencing; Mucinous cyst; Pancreatic cyst fluid; Pancreatic cancer

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Core Tip: Pancreatic cystic lesions are frequently found on imaging studies performed for other reasons, but differentiation between the different types and the detection of malignancy is often suboptimal with morphological features. Molecular analysis has been proposed to optimize cyst classification and the detection of malignancy. However, there is little evidence of its feasibility and usefulness in daily practice. The aim of this study was to evaluate the diagnostic yield of molecular analysis for the detection of mucinous and malignant cysts in routine clinical practice.

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INTRODUCTION

Pancreatic cysts are increasingly diagnosed as a consequence of both incidental

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findings on computed tomography (CT) and magnetic resonance imaging (MRI)^[1], and longer life expectancy of the population^[2,3]. Their diagnosis can generate a high degree of concern for both patients and physicians leading sometimes to the performance of multiple examinations, associated with an increase in diagnostic costs, and even unnecessary resections.

There are many different types of pancreatic cysts, including both neoplastic and non-neoplastic lesions. Their accurate diagnosis is important as some of them, such as mucinous cystic lesions or solid pseudopapillary tumors, are associated with a risk of malignancy, whereas others, such as serous cystic neoplasms and pseudocysts, are considered benign cysts. Mucinous cysts have a higher risk of malignant transformation. They can be divided into mucinous cystic neoplasm (MCN) and intraductal papillary mucinous neoplasm (IPMN), which can be branch-duct IPMN, mixed-type IPMN or main-duct IPMN (MD-IPMN). However, not all of them have the same risk of malignancy. According to recent publications, MCN have a 10%-17% risk of malignancy^[4,5], MD-IPMN 38%-68%^[2,6,7], branch-duct IPMN 12%-47%^[2,6], and solid pseudopapillary neoplasms 8%-20%^[6]. Furthermore, it is also important to note that the presence of an IPMN is associated with a higher risk of developing concomitant pancreatic adenocarcinoma^[8,9].

Pancreatic neoplasia is one of the most frequent causes of cancer-related death, with a 5-year survival lower than 10%^[9]. Only 20%-25% of pancreatic neoplasms are candidates for surgical treatment at diagnosis, and 80% of these will recur despite surgical intervention. Precursor lesions of pancreatic adenocarcinoma are pancreatic intraepithelial neoplasia and pancreatic cystic neoplasm (PCN)^[10], and their identification is crucial for early diagnosis and treatment, thus increasing survival of these patients.

Hence, the main diagnostic challenge for these lesions is the early detection of preneoplastic and malignant lesions, thereby avoiding unnecessary surgeries and establishing an adequate follow-up due to the risk of degeneration and the development of pancreatic adenocarcinoma. Therefore, an accurate diagnosis has prognostic, therapeutic and follow-up implications. Most PCN are incidentally detected in radiological tests performed for other reasons. However, in many cases it is difficult to differentiate between the different types of cysts and their risk of malignancy only by morphological characteristics, with an accuracy for adequate identification of the type of cyst of 40%-95% for MRI and 40%-81% for CT^[11].

Endoscopic ultrasound (EUS) is currently the diagnostic technique of choice for PCN as it allows not only assessment of morphological criteria, but also the performance of fine needle aspiration (FNA) and fluid analysis^[12]. Usually, cyst fluid analysis includes cytological and biochemical [carcinoembryonic antigen (CEA), and recently glucose] evaluation^[13-16]. However, accuracy for the diagnosis of mucinous cysts and malignancy detection remains suboptimal^[14,17]. There are different clinical practice guidelines for the diagnosis and treatment of PCN. The most commonly used are the International Association of Pancreatology guideline (IAP), the European guideline and the American Gastroenterological Association (AGA) guideline^[3,11,18]. However, the IAP and the European guidelines lead to unnecessary surgeries and the AGA to a decrease in sensitivity for the detection of malignancy^[5]. Therefore, multiple authors have evaluated the possibility of incorporating molecular analysis of cyst fluid for the diagnosis of pancreatic cysts, which has shown promising results^[14,19,20].

The aim of the current study was to determine the impact of molecular analysis on the detection of mucinous cysts and malignancy in routine clinical practice.

MATERIALS AND METHODS

This prospective trial was conducted in patients from a single center (Hospital Universitario de La Princesa, Madrid, Spain) over an 18-mo period.

Case selection

Consecutive patients over 18 years old referred to the Endoscopy Unit of Hospital Universitario de La Princesa with PCN and an indication for EUS-FNA following current clinical practice guidelines were recruited for the study. Inclusion criteria were: Lesions ≥ 15 mm in size, the need to confirm the diagnosis prior to surgical treatment, presence of worrisome features on imaging (wall thickening, main pancreatic duct > 5 mm, non-enhanced mural nodule, abrupt change in the size of the main pancreatic duct), changes on imaging during follow-up or an increase in serum CA 19.9. Patients were excluded from enrolment according to the following criteria:

Pregnancy, cysts with extra-pancreatic location or outside the scope of EUS, previous study with EUS-FNA, active treatment with anticoagulants or antiplatelets, thrombopenia (< 50.000 platelets/ μ L) or coagulopathy (INR < 1.5), or refusal to participate in the study. All participants enrolled in the study provided informed consent prior to the procedure. The study was approved by the Research Ethics Committee and prospectively registered on Clinical Trials (NCT03740360).

Imaging features prior to cyst fluid analysis

Radiological imaging impression was obtained by reviewing the radiological reports, and cysts were classified as malignant or without malignant features. A single endoscopist and anesthetist, both experts in their fields, performed the respective procedures in all study participants. All EUS were performed with a linear endoscopic ultrasound device (GF-UCT 180; Olympus Co., Japan). EUS features were described and recorded during the procedure, and lesions were classified as with or without worrisome features, and as malignant, mucinous or serous. After examination of the lesions contrast-enhanced EUS with Sonovue[®] (sulfur hexafluoride-filled microbubbles) was performed and the examination was recorded for later detailed re-evaluation. We defined three contrast patterns based on the cyst wall and septal enhancement: Hyper-enhanced, hypo-/iso-enhanced and mixed pattern.

Cyst fluid analysis

After antibiotic prophylaxis with 400 mg iv ciprofloxacin or 2 g ceftriaxone in the case of allergy to quinolones, cyst fluid was obtained by EUS-FNA with a 22 G needle (Expert Slimline, Boston[®]), and sent for cytologic, biochemical and molecular analysis. Both immediate and delayed (after 72 h) complications were registered.

Cytological evaluation: Smears were prepared on glass slides, 2/3 air-dried and 1/3 fixed in ethanol. Mucin staining with Alcian blue was performed on ethanol-fixed slides, and mucin detection was performed with the automatic Dakocytomation system (AR160). Lesions were categorized under Papanicolau classification and as mucin-staining positive or negative.

Biochemical analysis: At least 1 mL of cyst fluid was sent for analysis. We determined CEA levels in our laboratory with the Architect system by chemiluminescent immunoassay. Following prior studies, the CEA cut-off point was established as 192 ng/mL to differentiate mucinous (< 192 ng/mL) from non-mucinous. From the 16th lesion included in the study, intracystic glucose determination was added to the protocol, as recent evidence indicates that glucose levels < 50 mg/dL are suggestive of mucinous cysts^[15,16]. Glucose determination was performed in our Hospital laboratory (using calibration for the determination of glucose in biological fluids).

Molecular analysis: After cytological and biochemical analysis, the excess fluid was frozen and stored at -80°C until all patients were recruited. The range of volumes available for molecular analysis was 0.3-5 mL. The collection was registered in the Spanish National Register of Biobanks of the Carlos III Health Institute. The genomic deoxyribonucleic acid (DNA) present in the pancreatic cyst fluid was manually purified using the NZY Blood gDNA Isolation kit (NZYtech) following the manufacturer's recommendations. The extracted DNA was fluorimetrically quantified using the Quantus (Promega) system. The integrity of the DNA obtained was determined in the Agilent 2100 Bioanalyzer (Agilent) using the Agilent High Sensitivity DNA (Agilent) kit. Due to the low concentrations obtained in some samples, DNA was concentrated up to a concentration of 30 ng/ μ L, using magnetic beads (AMPure XP beads, Beckman Coulter). Although cyst fluid was initially obtained from the 36 pancreatic cysts, only 25 of them yielded the amount of DNA needed to perform sequencing (100 ng of DNA at a concentration of 30 ng/ μ L). The targeted Next-Generation Sequencing (tNGS) was performed in the MiSeqTM platform (Illumina) using a panel designed specifically for this project (Roche).

Gene panel bioinformatic design

All exons of the following genes were included and sequenced by tNGS: *AKT1*, *ALK*, *APC*, *BRAF*, *CDKN2A*, *CDH1*, *CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ESR1*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FOXL2*, *GNA11*, *GNAQ*, *GNAS*, *HRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *NOTCH1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PIK3R1*, *PTEN*, *RET*, *RNF43*, *ROS1*, *SMAD4*, *TGFBR2*, *TP53*, *VHL*. Therefore, coverage was complete.

NGS data analysis

Coverage analysis showed that nearly 100% of the regions were covered at a depth of 100 × or more in all the samples, reaching 400 × in a very high percentage of them. The search for variants was carried out with the VarScan software (<http://varscan.sourceforge.net/>). Among the variants identified, approximately 400, those present in more than 75% of the samples which did not appear as mutations noted in the databases were excluded as they were not likely to participate in the development of the disease. Variants with very low frequency (< 1 reading) were eliminated from the study since these could be due to errors in sequencing. For the final analysis, the variants detected with a frequency between 1%-33% were included. A total of 78 variants were detected in the 25 samples analyzed (mean of 3 mutations per sample). Comparisons between samples and identification of the pathogenicity of variants were carried out using the PredictSNP2tool (<https://Loschmidt.chemi.muni.cz/predictsnp2/referencia>). In addition, the information from the predictive tools was combined with the results of the search in the ClinVar database, which contains the interpretation of the relationship between variants and their significance for human health.

Diagnostic criteria for malignant/benign cysts

We defined as malignant those PCN that met any of the following criteria^[21]: EUS-FNA cytology suspicious or compatible with malignancy; High-grade dysplasia or invasive carcinoma in the histology analysis of a surgical specimen; Progression of the PCN and/or metastatic disease in the imaging tests during follow-up; Death related to neoplastic complications up to 6 mo after diagnosis; Clinical follow-up consistent with underlying tumor disease for 6 mo.

In the absence of a definitive histopathological diagnosis, we defined a "pseudo-gold standard" to classify lesions into mucinous and non-mucinous (Figure 1), based on the previous evidence and the recommendations of clinical practice guidelines^[6,10,11,22].

Variables

The following data were recorded for each patient: Age, sex, American Society of Anesthesiologist classification, treatment with antiplatelets or anticoagulants, history of pancreatitis, neoplasia, smoking or familial pancreatic cancer, presence of symptoms, radiological diagnosis, date of EUS examination, EUS diagnosis, complications, size and location of the lesions, biochemical, cytological and molecular analysis of cyst fluid, histopathological diagnosis in the case of surgery, follow-up and diagnosis of malignancy following the above-mentioned criteria.

Statistical analysis

Continuous variables are expressed as average ± SD and were compared between groups using the Student's *t*-test or *U* Mann-Whitney test. Categorical variables are expressed as percentage, and comparisons were made with the χ^2 or Fisher's exact test. The level of agreement reached was determined with Cohen's kappa. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of diagnostic variables were determined according to the pseudo-gold standard established in the study. *P* values ≤ 0.05 were considered significant. All the statistical analyses were performed with the IBM SPSS 23.0 or Stata v13.0 program.

RESULTS

Patient baseline characteristics

Eighty-seven patients with a total of 95 PCN were included between June 2017 and December 2018. After applying the exclusion criteria, 52 patients with 59 PCN were excluded: 47 lesions < 15 mm in size, 6 patients with 6 PCN did not agree to participate, 4 due to lack of modification of the plan following the results of EUS-FNA, and 2 lesions due to lack of technical safety to reach the lesion. In one of these cases access was limited by interposition of gastric neoplasia. Thus, 35 patients with 36 PCN were initially enrolled. Demographic and clinical characteristics are detailed in Table 1.

Lesion characteristics

Table 2 summarizes the lesion characteristics on radiological (CT and MRI) and EUS examinations. None of the 8 mural nodules detected on EUS were described in the

Table 1 Demographic and clinical data of the study population, *n* (%)

Patients	<i>n</i> = 35
Age (yr)	66.7 ± 14.5
Male gender	17 (48.6)
ASA I-II	25 (71.4)
AAS	5 (14.3)
Smoking	12 (34.3)
History of acute pancreatitis	3 (8.6)
History of extrapancreatic neoplasia	10 (28.6)
Family history of pancreatic cancer	3 (8.6)
Symptoms	10 (28.6)

Quantitative variables are expressed as mean and standard deviation. Qualitative variables are expressed as absolute values; percentages are indicated in parentheses. ASA: American Society of Anesthesiologist classification; AAS: Acetylsalicylic acid.

radiological imaging techniques. **Table 3** summarizes the results of cyst fluid analysis. CEA levels were not determined in 7/36 (19.4%) PCN due to technical problems associated with the high viscosity of the fluid ($n = 1$; 14.3%) or insufficient sample ($n = 6$; 85.7%). In the case of glucose levels, they could not be determined in 6/22 (27.3%) because of high viscosity ($n = 1$; 16.7%) or insufficient sample ($n = 5$; 83.3%).

Lesion classification

Classification of 2 (5.6%) of the PCN into mucinous or non-mucinous lesions was not possible because the mucin stain was negative and no additional CEA or glucose was available. The remaining 34 lesions were classified following the algorithm described in **Figure 1**. Twenty-eight (82.4%) were classified as mucinous because they met at least one of the criteria and 6 (17.6%) as non-mucinous.

Molecular analysis for the identification of mucinous cystic lesions and malignant cysts

The mean volume of liquid sent for molecular analysis was 2.1 ± 2.3 mL. Although cyst fluid was initially collected from the 36 PCN, only 25 (69.4%) had the amount of DNA needed to perform sequencing (100 ng of DNA at a concentration of 30 ng/ μ L). The cyst fluid volume obtained for molecular analysis in the cases with enough DNA was lower (1.8 ± 1.8 mL) compared to those with insufficient DNA (2.7 ± 3.1 mL). No statistically significant relationship was found between cyst fluid volume and the possibility of performing molecular analysis.

The results of molecular analysis are shown in **Table 4**. Overall, mutations in *KRAS* were found in 16 (64%) cysts, *GNAS* in 13 (52%), *PIK3R1* in 1 (4%), *IDH1* in 1 (4%), *PDGFRA* in 3 (12%), *FGFR3* in 2 (8%), *RET* in 1 (4%), *ERBB2* in 1 (4%), *BRAF* in 1 (4%), *TGFBR2* in 1 (4%), *FBXW7* in 1 (4%) and *MAP2K1* in 1 (4%) cyst. No mutations were found in the other genes analyzed.

Molecular analysis was possible in 18/28 (64.3%) of the cysts classified as mucinous and in 5/6 (83.3%) of the lesions classified as non-mucinous. In addition, sufficient DNA was obtained in two lesions that could not be classified as mucinous or non-mucinous using the cytological and biochemical criteria described in the previous section.

Mucinous cystic neoplasms: None of the mutations were associated with mucinous cysts ($P > 0.05$). Mutations in *KRAS* and *GNAS* were found in 13/18 (72.2%) and 10/18 (55.6%) of the cysts classified as mucinous, respectively. *KRAS* had an 81.2% sensitivity (95%CI: 59-100) and 71.4% specificity (95%CI: 30.9-100) ($P = 0.297$), while *GNAS* had a 76.9% sensitivity (95%CI: 50.1-100) and 80% (95%CI: 50.2-100) specificity ($P = 0.640$) for mucinous cyst diagnosis. When combining *KRAS* and *GNAS* mutations, 15/18 (83.3%) of the mucinous cysts presented mutations in *KRAS* and/or *GNAS*, offering an 83.3% sensitivity (95%CI: 63.3-100), 60% specificity (95%CI: 7.06-100), 88.24% PPV (95%CI: 69.98-100) and 50% NPV (95%CI: 1.66-98.34) ($P = 0.086$) for the detection of mucinous cysts.

Table 2 Morphological characteristics

Radiological imaging tests	n (%)
Malignant	7 (19.4)
Non-malignant	29 (80.6)
Worrisome features on EUS	18 (50)
EUS diagnosis	
Malignant	4 (11.1)
BD-IPMN	9 (25)
MD-IPMN	14 (38.9)
MCN	5 (13.9)
SCN	4 (11.1)
Location	
Head	21 (58.3)
Body	15 (41.7)
Tail	0
Multifocal	8 (22.9)
Size (mm)	27 ± 15.5
Size MPD > 3 mm	11 (30.6)
Mural nodule	8 (22.2)
Contrast enhancement pattern	
Hypo/iso-enhanced walls	18 (54.5)
Hyperenhanced walls	12 (36.4)
Mixed enhancement pattern	3 (9.1)

Quantitative variables are expressed as mean ± standard deviation. Quantitative variables are expressed as absolute values, and their proportions are in bracketed text. EUS: Endoscopic ultrasound; BD-IPMN: Branch duct intraductal papillary mucinous neoplasm; MD-IPMN: Main duct intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm; SCN: Serous cystic neoplasm; MPD: Main pancreatic duct.

Non-mucinous cystic neoplasms: In a similar manner to mucinous cysts, none of the detected mutations were statistically associated with non-mucinous cyst diagnosis. Mutations in *KRAS* and *GNAS* were found in the same 2/5 (40%) PCN; therefore, the combination of both mutations did not provide different results.

Undetermined cystic lesions: Molecular analysis was also performed in 2 (5.6%) PCN that could not be classified as mucinous or non-mucinous. One of them had mutations in *KRAS* and *GNAS*, while no mutations were found in the other cyst.

Malignant cystic neoplasms: Molecular analysis was carried out in 4/5 (80%) of the malignant lesions and in 21/31 (67.7%) of the non-malignant lesions. Mutations in *KRAS* and/or *GNAS* were found in the 4 (100%) lesions classified as malignant and in 14/21 (66.7%) of the non-malignant lesions. No mutations in *PIK3CA* were found in any of the malignant cysts analyzed. None of the mutations found were related to malignancy ($P > 0.05$).

DISCUSSION

In this study, we evaluated the diagnostic yield of molecular analysis of cyst fluid obtained by EUS-FNA for mucinous cyst diagnosis and the detection of malignancy.

Previous studies have shown that mutations present in the histopathological analysis of pancreatic tissue obtained from surgical specimens are also present in pancreatic cyst fluid, although the amount of DNA obtained from fluid analysis is

Table 3 Cyst fluid analysis

Biochemical	n (%)
CEA (<i>n</i> = 29)	
< 192 ng/mL	14 (48.3)
≥ 192 ng/mL	15 (51.7)
Glucose (<i>n</i> = 16)	
< 50 mg/dL	10 (62.5)
≥ 50 mg/dL	6 (37.5)
Cytological	
Papanicolaou classification (<i>n</i> = 36)	
II	13 (36.1)
IV	22 (61.1)
VI	1 (2.8)
Mucin staining (<i>n</i> = 36)	
Positive	22 (61.1)
Negative	14 (38.9)
Molecular	
Possible	25 (69.4)
Not possible	11 (30.6)

Quantitative variables are expressed as absolute values, and their proportions are in parentheses. CEA: Carcinoembryonic antigen.

lower and sometimes insufficient for molecular analysis^[23,24]. In our series, we obtained enough material to perform the molecular analysis (100 ng of DNA at a concentration of 30 ng/μL) in 69.4% of included PCN. An insufficient amount of intracystic DNA was not associated with a lower volume of fluid obtained. These results are similar to those reported in previous studies, which described that the volume required to perform molecular analysis ranges between 0.2-0.5 mL, although in some samples the amount of DNA is insufficient to perform the analysis^[23,25,26]. Therefore, we assume that the amount of intracystic DNA is low, and in some cases it may be insufficient to perform molecular analysis, providing negative results regardless of cyst fluid volume.

tNGS detected the following mutations: *KRAS* in 16 (64%) cysts, *GNAS* in 13 (52%), *PIK3R1* in 1 (4%), *IDH1* in 1 (4%), *PDGFRA* in 3 (12%), *FGFR3* in 2 (8%), *RET* in 1 (4%), *ERBB2* in 1 (4%), *BRAF* in 1 (4%), *TGFBR2* in 1 (4%), *FBXW7* in 1 (4%) and *MAP2K1* in 1 (4%) cyst. No mutations were found in the rest of the evaluated genes. These results are in accordance with those of Jones *et al*^[19], who evaluated 92 pancreatic cysts by tNGS for the presence of mutations in 39 genes; they found no mutations in 43% of the included cysts and the most frequently detected mutations, as in our series, were *KRAS* and *GNAS*. In order of decreasing frequency, mutations were found in the following genes: *KRAS* (47%), *GNAS* (24%), *CDKN2A* (6%), *VHL* (2%), *SMAD4* (1%) and *TP53* (1%). We found mutations in *KRAS* in 72.2% and *GNAS* in 55.6% of mucinous lesions. When combining these results, 83.3% of mucinous cysts harbored a mutation in one or both genes. However, neither *KRAS* nor *GNAS* or other genes were related to mucinous cyst diagnosis ($P > 0.05$). Regarding the lesions classified as non-mucinous, mutations were found in *KRAS* in 40% of these lesions and in *GNAS* in the same 40%. Similar to mucinous cysts, none of the mutations were related to non-mucinous cyst diagnosis ($P > 0.05$). We did not find any mutations in *VHL*. However, although its presence has been related to serous cystic neoplasms with high specificity, the frequency of this mutation is low. Jones *et al*^[19] analyzed fluid from 92 PCN using NSG and found *VHL* mutations in 2% of them. Springer *et al*^[20] found mutations in 42% of histopathologically confirmed serous cystadenomas, although they carried out their determination in cyst fluid obtained from surgical specimens and therefore, the percentage could be higher.

Some authors have raised the possibility of incorporating molecular analysis of PCN due to the high specificity of *KRAS* and *GNAS* for mucinous cysts diagnosis found in

Table 4 Molecular analysis

	Non-mucinous				Mucinous																		
	PCN5	PCN15	PCN18	PCN20	PCN33	PCN0	PCN1	PCN2	PCN3	PCN4	PCN7	PCN11	PCN13	PCN14	PCN16	PCN17	PCN19	PCN21	PCN24	PCN25	PCN29	PCN30	PCN34
<i>KRAS</i>	M	M	N	N	M	M	N	N	N	N	M	N	N	N	N	M	N	N	N	M	M	N	N
<i>GNAS</i>	M	M	N	N	M	N	N	N	N	N	N	N	M	N	N	M	M	N	M	M	M	M	M
<i>VHL</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>P53</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>PIK3R1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	N	M	M	M
<i>EGFR</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>ALK</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>NOTCH1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>GNA11</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>CDKN2A</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>APC</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>FGFR2</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>IDH1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	N	M	M	M	M	M	M	M
<i>PIK3CA</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>KIT</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>MET</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>FGFR1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>ROS1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>GNAQ</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>PDGFRA</i>	M	M	M	M	M	M	M	M	N	M	M	M	M	M	M	M	M	M	M	N	M	N	
<i>FGFR3</i>	N	M	M	M	M	M	M	M	M	M	M	M	N	M	M	M	M	M	M	M	M	M	M
<i>RNF43</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>RET</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	N
<i>ERBB2</i>	M	M	M	M	M	M	M	M	N	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>DDR2</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M

<i>BRAF</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	N	M
<i>ESR1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>FGFR2</i>	M	M	M	M	M	M	N	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>FBXW7</i>	M	M	N	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>FOXL2</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>MAP2K1</i>	M	M	M	M	M	M	N	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>AKT1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>CTNNB1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>SMAD4</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>PTEN</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>NRAS</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>IDH2</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>HRAS</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
<i>CDH1</i>	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M

PCN: Pancreatic cystic neoplasm. M: Mutated; N: Not-mutated.

previous studies with histopathological correlation, and the small volume required for their determination^[12,19,27,28]. Nikiforova *et al*^[26] performed molecular analysis of cyst fluid obtained by EUS-FNA and found that the presence of a *KRAS* mutation offered a sensitivity of 54% and specificity of 100% for mucinous cyst diagnosis^[29]. Similarly, Amato *et al*^[24] described that *KRAS* and/or *GNAS* were mutated in 92% of *IPMN*, *GNAS* in 79%, *KRAS* in 50% and both in 37.5%^[30]; Singhi *et al*^[23] found mutations in *GNAS* in 39%, *KRAS* in 68% and both in 83% of *IPMN*, although only 6% of the *MCN* had mutations in *KRAS* and/or *GNAS*^[31]. Al-Haddad *et al*^[32] found that the presence of a mutation in *KRAS* and/or ≥ 2 loss of heterozygosity in cyst fluid obtained by EUS-FNA demonstrated 50% sensitivity and 80% specificity for the diagnosis of mucinous cysts. In their study, 58% of the mucinous cysts with histopathological diagnosis did not present *KRAS* mutations. However, molecular analysis allowed adequate classification of 24% of the mucinous cysts that could not be classified by CEA and cytological analysis. In this study, *KRAS* offered 81.2% sensitivity and 71.4% specificity, *GNAS* 76.9% sensitivity and 80% specificity, and the combination of *KRAS* with *GNAS* 83.3% sensitivity and 60% specificity for the diagnosis of mucinous cysts. Our sensitivity is close to or higher than that of the studies described above, even in those where the fluid was obtained by aspiration of the surgical specimen. On the

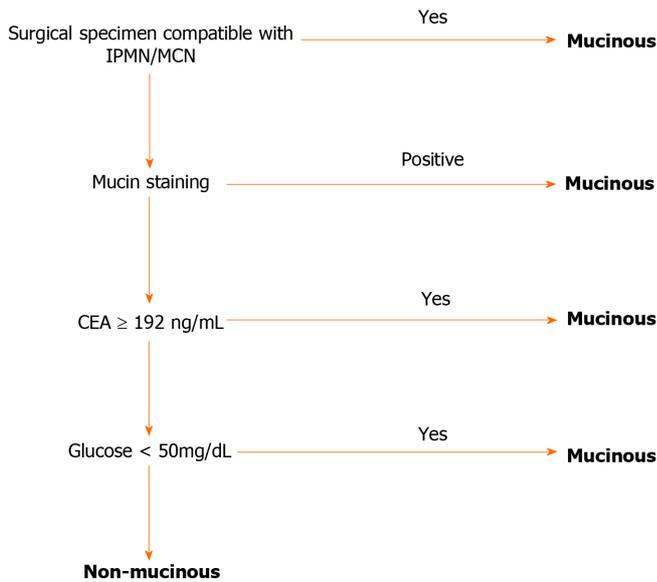


Figure 1 Diagnostic algorithm for mucinous and non-mucinous cysts. Pseudogold standard was considered positive (mucinous) if: Mucinous histology and/or positive mucin staining and/or biochemical > 192 ng/dL and/or glucose < 50 mg/dL, whereas it was considered negative (non-mucinous) if: Non-mucinous histology, negative mucin staining, biochemical < 192 ng/dL and glucose > 50 mg/dL. IPMN: Intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm; CEA: Carcinoembryonic antigen.

other hand, our specificity was lower due to the absence of histopathological correlation in some lesions, which could have modified the final diagnosis, and the smaller population of our series.

Taking into consideration the high specificity of *KRAS* and *GNAS* in previous studies for the diagnosis of mucinous cysts^[26], the 2 (40%) lesions without histopathological diagnosis classified as non-mucinous would have been recategorized as mucinous after molecular analysis due to the presence of mutations in both *KRAS* and *GNAS*. This would have led to a modification of the follow-up plan in 8% of the cysts in which molecular analysis was successfully performed. Additionally, of the 2 indeterminate cysts in our study, one showed mutation in both *KRAS* and *GNAS* so it could have been classified as mucinous. Therefore, we agree that performing molecular analysis, at least in selected cases with uncertain diagnosis, could improve diagnosis by adequately categorizing PCN as mucinous. This is important as mucinous cysts are premalignant lesions and have a higher risk of concomitant pancreatic adenocarcinoma, thus implying long-term follow-up. We agree with the statement made by other authors about the usefulness of associating the determinations of CEA (more sensitive) and *KRAS*/*GNAS* (more specific)^[24,33]. However, further prospective studies with histopathological correlation are needed.

Another area of interest in molecular analysis is the detection of malignancy given the low diagnostic accuracy of other diagnostic methods for early detection of malignant PCN and the morbimortality associated with pancreatic surgery. In our case we were able to evaluate the presence of mutations in 80% of malignant lesions. We found mutations in *KRAS* and/or *GNAS* in all (100%) malignant lesions, but none of these lesions showed mutations in *PIK3CA*. Additionally, we found mutations in *IDH1* ($n = 1$) and *TGFBR2* ($n = 1$). In our series no mutations were statistically related to malignancy ($P > 0.05$). Similarly, in previous studies *KRAS* and *GNAS* have not been related to malignancy and have been described as mutations that occur in the early stages of pancreatic carcinogenesis^[10,19,25]. In contrast, other mutations such as *TP53*, *PIK3CA*, *PTEN* or loss of *SMAD4* have been associated with malignancy^[10,19,23,34]. Our results, similar to those obtained in the study by Singhi *et al.*^[23], show that *KRAS* and *GNAS* are mutations that occur in the early stages of carcinogenesis and are therefore present in 100% of malignant mucinous cystic neoplasms. However, they found that 50% of the IPMNs with high grade dysplasia and 100% of the IPMNs with adenocarcinoma had, in addition to the *KRAS* and/or *GNAS* mutations, mutations in *TP53*, *PIK3CA* and/or *PTEN*. In our study we found no mutations in *TP53*, *PIK3CA* or *PTEN*. These differences could be justified by the low incidence of malignancy in our sample, differences in the time from extraction to the performance of the molecular analysis and differences in the process of molecular analysis.

There are several clinical practice guidelines focused on diagnosis, treatment and

follow-up of PCN, with differences in the indication of EUS-FNA, surgery and follow-up^[2,3,11,18,22,35-37]. These differences show the lack of agreement regarding the role and indication of this technique, probably due to the challenge of early detection of malignancy combined with avoiding unnecessary surgeries. They also reflect disagreement in establishing cost-effective follow-up strategies. The AGA guideline has been widely criticized for its low diagnostic accuracy for detection of malignant cystic lesions, and for its recommendation to discontinue long-term follow-up in the absence of significant findings or changes^[12,38,39]. In addition, the European guideline and the IAP guideline have also been criticized mainly for the high number of unnecessary surgeries related to their recommendations^[5,40]. Therefore, several authors have proposed alternative algorithms based mainly on lowering the threshold for the indication of EUS-FNA and on performing molecular analysis^[12,41,42].

According to the European guideline^[11], we believe it is advisable to continue follow-up in mucinous lesions, while it could be discontinued in serous cysts. However, differentiation between serous and mucinous PCN is difficult, so the European guideline advises performing EUS-FNA with cytological analysis, CEA and molecular analysis (NGS) with determination of *KRAS* and *GNAS* when the diagnosis is unclear^[11]. In contrast, the IAP guideline considers that molecular analysis is experimental and should only be considered in centers with experience in this technique^[9]. We have proven that the performance of molecular analysis is a complex procedure, with high cost and requires an experienced team; thus, we consider, in line with IAP guidelines, that the technique should be standardized before recommending its widespread use.

The main strengths of our study are its prospective nature with a cohort of patients with different types of PCN (82.4% mucinous and 17.6% non-mucinous cysts) and malignancy (13.8%), which shows the standard clinical practice in the study and therapeutic decision on PCN, and therefore our experience is applicable to clinical practice in any other center with access to pancreatic study techniques. Additionally, we performed molecular analysis providing additional information on PCN diagnosis.

However, our study has several limitations. First of all, it is a unicentric study based on the experience of a single endoscopist. Second, it should be noted that the diagnosis using morphological, cytological and biochemical criteria is suboptimal and we only have anatomopathological diagnosis in 5 (13.9%) of the lesions. In fact, as we have already discussed, in 2 lesions classified as non-mucinous, initial diagnosis would have been modified after performing molecular analysis. We consider that our system of classifying the PCN is a good option in clinical practice, where the diagnosis is made with the available data in the absence of a surgical specimen. Third, the absence of malignancy was defined as the absence of progression in imaging tests or clinical deterioration after a follow-up of no less than 6 mo, being the median follow-up in our study of 472 (IQR: 271-619) d. However, the follow-up period could be considered short and it is uncertain if patients could have developed malignancy over a longer follow-up period. Fourth, the small sample size of the study, which was due to the short temporal frame of the study and inclusion criteria, resulted in the absence of statistical significance. Only lesions ≥ 15 mm were included following the recommendations of the European guideline^[11], excluding those < 15 mm, even though the presence of malignancy was described in up to 39% of the symptomatic cysts < 2 cm^[43]. Finally, we emphasize that, although we consider that molecular analysis is highly specific for the diagnosis of mucinous cysts, the high cost of this technique precludes its universal implementation.

CONCLUSION

In conclusion, molecular cyst fluid analysis obtained by EUS-FNA helped in our study by recategorizing 40% of serous lesions as mucinous cysts. However, the mutations detected in our sample did not reach statistical significance for the diagnosis of mucinous or malignant cysts. Further studies with larger sample sizes and more sensitive techniques could change these results.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cysts are a common finding on imaging tests performed for other reasons.

Adequate characterization is important considering the risk of malignancy of some of these cysts. However, differentiation between different types of cysts and detection of malignancy just with morphological criteria is suboptimal.

Research motivation

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) and molecular analysis could improve the detection of mucinous (pre-malignant) and malignant cysts.

Research objectives

To determine the diagnostic yield of molecular analysis for the detection of mucinous and malignant cysts in clinical practice.

Research methods

A single center, prospective observational study of consecutive patients over an 18-month period with pancreatic cystic lesions and an indication for EUS-FNA following European clinical practice guidelines was conducted. EUS-FNA with cytological, biochemical with CEA and glucose, and molecular analysis with next-generation sequencing were performed in 36 pancreatic cysts. Next-generation sequencing results were compared for cyst type and malignancy.

Research results

Of the 36 lesions included, 28 (82.4%) were classified as mucinous and 5 (13.9%) lesions as malignant. The amount of DNA obtained was sufficient for molecular analysis in 25 (69.4%) pancreatic cysts. *KRAS* and/or *GNAS* showed 83.33% sensitivity, 60% specificity, 88.24% PPV and 50% NPV ($P = 0.086$) for the diagnosis of mucinous cystic lesions. Mutations in *KRAS* and *GNAS* changed the follow-up plan in 8% of the cysts. None of the mutations analyzed were related to malignancy ($P > 0.05$).

Research conclusions

Molecular cyst fluid analysis obtained by EUS-FNA improved mucinous cyst diagnosis by recategorizing 40% of serous lesions as mucinous cysts. However, the mutations detected in our cohort did not reach statistical significance to confirm the diagnosis of mucinous or malignant cysts.

Research perspectives

Further prospective studies with larger sample sizes are needed to determine the clinical benefit of adding molecular cyst fluid analysis for pancreatic cyst evaluation.

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