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Biomarkers for classification and risk assessment of pancreatic cystic neoplasms

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"If I have seen further it is by standing on the shoulders of giants."
Isaac Newton

Preface

My first clinical contact with pancreatic cystic lesions (PCLs) took place at Medical University of South Carolina (MUSC) in 2004, during a one-year hands-on training in Endoscopic Ultrasound (EUS). During those busy EUS days, which typically included 6 to 8 procedures, whenever there was a putative diagnosis of a pancreatic cyst for EUS evaluation, an additional informed consent had to be obtained to collect PCF samples for the multicenter PANDA study.[1] This study, published in 2009, pioneered the evaluation of genetic mutations for pancreatic cyst diagnosis. For its accomplishment, 391 patients were recruited in seven centers over a two-year period from 2004 to 2006.

In this initial contact with prospective trials, I understood that even in referral centers only one or two patients at most were included each week. Patient inclusion was unproductive, as patients with cysts were rare in those days and some refused to give consent for the study. Even after overcoming these issues, often the cysts did not require fine needle aspiration (FNA) or after a FNA successfully performed, no remnant pancreatic cystic fluid (PCF) was available for the study following standard analysis.

Finished an amazing year dedicated to EUS learning, it was time to return to the *Instituto Português de Oncologia de Lisboa de Francisco Gentil, EPE* (IPOLFG, EPE). After that experience abroad, an early decision was to create a prospective EUS database of pancreatic cystic lesions (PCLs) and a biorepository of PCF samples. Cystic lesions were obvious candidates due to diagnostic uncertainty, the simplicity of freezing the PCF leftover, without standard analysis precluded and no additional procedure required for sample collection.

Besides the experience abroad, there was another essential protagonist – his name was Ruben Roque, MSc - a particularly devoted cytology technician. A first attempt to start the PCF biorepository ended with all the samples collected going to waste, due to an undisclosed freezer appraisal. In the second attempt, Ruben collaborated with his organizational skills, centralized the specimen storage, optimized the PCF collection protocol, started the plasma storage protocol, and kept the database of the biorepository updated. Additional persistence, curiosity, mentorship, focus, and hope helped to propel this doctoral dissertation forward.

This dissertation addresses translational research for diagnosis of PCLs, which are known precursors of pancreatic ductal adenocarcinoma (PDAC). It specifically evaluates different biomarkers to optimize the diagnosis of pre-malignant, high-risk (high-grade dysplasia or adenocarcinoma), and other malignant cysts, such as cystic neuroendocrine tumors (NETs).

It is organized in seven chapters: I. Introduction; II. Rationale and Research Questions; III. Material and Methods; IV. Results; V. Discussion; VI. Conclusions and Final Remarks.

The first three chapters include a literature review, followed by the presentation of the rationale and research questions, and a general description of the methodology used in the experimental work.

The fourth chapter includes publications accepted or submitted for publication, clustered in three thematic sets in the results section. The first set presents a literature review of molecular analysis and the second and third sets analyze ancillary studies for diagnosis of PCLs.

The first thematic set, Molecular Analysis for Assessment of Pancreatic Cysts, includes two original metanalyses that were planned within the framework of the *Grupo de Revisões Sistemáticas da Universidade da Beira Interior* (GRUBI): 1) *"Faias S, et al. KRAS in cyst fluid obtained by EUS-FNA in pancreatic cystic lesions: A systematic review and meta-analysis. Pancreas 2019;48 (6):749-758"* and 2) *"Faias S et al. Accuracy and diagnostic yield of genetic testing versus microforceps biopsy for diagnosis of pancreatic cysts: a systematic review and meta-analysis. World J Gastroenterol; 2019; 25 (26):3450-346",* both published. These studies helped to expand the knowledge on the topic and to introduce the theme. They refer to *KRAS* mutational analysis in PCF and to genetic testing *versus* histology of cystic wall, obtained with microforceps biopsies, for the diagnosis of PCLs.

The second thematic set on Biomarkers for Diagnosis of Mucinous Pancreatic Cysts includes two original papers: 1) *"Faias S, et al. Clinical impact of KRAS and GNAS analysis added to CEA and cytology in pancreatic cystic fluid obtained by EUS-FNA. Dig Dis Sci. 2018; 63(9):2351-2361"* and 2) *"Faias S, et al. Excellent accuracy of glucose level in cystic fluid for diagnosis of pancreatic mucinous cysts. Dig Dis Sci. 2019 Nov 9. Doi: 10.1007/s10620-019-05936-5".*

The third thematic set on Biomarkers for Diagnosis of High-risk and Malignant Pancreatic Cysts includes four original papers: 1) "*Faias S, et al. A second EUS-FNA for cytology identifies high-risk pancreatic cysts overlooked by current guidelines",* accepted for publication; 2) "*Faias S, et al. Endoscopic Ultrasound with Fine Needle Aspiration is useful in pancreatic cysts smaller than 3 cm"*, submitted for publication; 3) "*Faias S, et al. Chromogranin A and NSE in pancreatic cystic fluid are useful biomarkers for diagnosis of cystic pancreatic neuroendocrine tumors*", submitted for publication; and 4) "*Faias S, et al. Methylation changes at the GNAS imprinted locus in pancreatic cystic neoplasms are important for the diagnosis of malignant cysts",* submitted for publication.

The final chapters include a unifying discussion and conclusion with final remarks including an improved diagnostic flowchart designed in accordance with the conclusions of these publications, in order to better differentiate relevant cysts in the myriad of incidental PCLs, which currently is like trying to find a needle in a haystack.

Ars longa, vita brevis, occasio praeceps, experimentum periculosum, iudicium difficile.

"A arte é longa, a vida breve, a ocasião fugaz, a experiência perigosa, o julgamento difícil."
"The art is long, life short, opportunity fleeting, experience dangerous, judgement difficult."

Hipócrates, 400 AC

Resumo

As lesões quísticas pancreáticas (PCL) têm incidência crescente devido ao envelhecimento da população e ao aumento da utilização dos métodos de imagem. Na prática clínica pretende-se distinguir os quistos mucinosos, de alto risco e malignos, que requerem tratamento cirúrgico, dos quistos benignos ou pré-malignos de baixo risco, que no máximo requerem vigilância. O objetivo do presente trabalho é analisar de forma abrangente, biomarcadores em líquido de quisto pancreático (PCF) obtido por Ecoendoscopia com punção (EUS-FNA), numa coorte de quistos predominantemente de baixo risco sob vigilância imagiológica, que são os mais comuns na prática clínica.

A análise de PCF nesta coorte inclui estudos de genómica (mutações no DNA), epigenómica (análise de metilação), metabolómica (glicose) e proteómica (CEA, cromogranina A, NSE), com avaliação de biomarcadores para diagnóstico de quistos mucinosos e quistos malignos, que beneficiam de vigilância e ressecção cirúrgica, respetivamente.

Numa primeira meta-análise comparámos a metodologia diagnóstica atual - CEA e citologia - com as mutações do *KRAS* para diagnóstico dos quistos mucinosos. O CEA foi o melhor teste em quistos clinicamente significativos (AUC=0.69), e a citologia em quistos malignos (AUC=0.78), superando as mutações do KRAS (AUC=0.53 e AUC=0.56, respetivamente). Numa segunda meta-análise comparámos a precisão diagnóstica da análise molecular *versus* biópsia com micropinça (MFB) no diagnóstico de PCL. As duas abordagens foram idênticas em quistos benignos, mas a análise molecular foi superior em quistos mucinosos tanto de baixo como de alto risco.

Além das duas meta-análises, realizámos um estudo retrospetivo para avaliar o valor das mutações do *KRAS* e do *GNAS* em 52 amostras de PCF congeladas. Concluímos que não têm valor adicional no diagnóstico diferencial das PCL, relativamente aos testes convencionais. Noutra publicação comparámos o nível de glicose em PCF com o CEA para diagnóstico de quistos mucinosos em 82 doentes. O CEA >192 ng/ml apresentou uma AUC de 0.84 e a glicose <50 mg/dl de 0.86. Além da maior precisão diagnóstica, a glicose avaliada *in loco* com um glicosímetro, é fácil, imediata e requer um volume mínimo de PCF.

No estudo seguinte, avaliámos se uma segunda EUS-FNA alterou o diagnóstico ou a decisão de quistos pancreáticos. Comparámos 105 doentes com uma única EUS-FNA com 23 doentes com uma segunda EUS-FNA. Esta pode ser recomendada, pois cerca de 20% dos doentes foram referenciados para cirurgia após repetição da EUS-FNA, incluindo dois com tumores neuroendócrinos (NET) quísticos. Seguidamente, explorámos o papel da EUS-FNA em pequenas PCL (<3 cm), num estudo com 115 PCL <3 cm. 19/115 foram operadas, correspondendo a 15 lesões malignas ou pré-malignas e 4 benignas. Concluímos que a EUS-FNA em quistos com <3 cm pode melhorar o diagnóstico e o custo-efetividade, pois confirmou

malignidade em lesões ressecadas, e diagnosticou quistos benignos que podem ser libertados de vigilância. Num estudo piloto com 16 doentes, incluindo 4 com NET quísticos, avaliámos o valor diagnóstico da cromogranina A (CroA) e da enolase específica neuronal (NSE) em PCF. Os níveis de CroA e NSE foram mais elevados nos NET quísticos, com uma AUC de 0.94 para a CroA e 1 para a NSE. Estes revelaram-se biomarcadores promissores

Por fim, estudámos alterações epigenéticas no diagnóstico de quistos malignos. Analisámos a metilação do *locus GNAS* em PCF para perceber se se associa à progressão maligna de PCL. Estudámos 52 amostras e observámos que a alteração da metilação se associou significativamente a malignidade. Trata-se do primeiro trabalho a avaliar alterações de metilação no *locus GNAS* no diagnóstico de PCL.

Terminamos este trabalho com uma proposta de revisão do organograma de diagnóstico das PCL baseado nas *guidelines* atuais, que incorpora os resultados desta tese.

Palavras-chave

Quisto do pâncreas; Ecoendoscopia com punção com agulha fina (EUS-FNA); Líquido quístico pancreático; CEA; Citologia; Análise Molecular; Biomarcador

Resumo Alargado

As lesões quísticas pancreáticas (PCL) são achados imagiológicos incidentais com frequência crescente devido ao envelhecimento da população e ao aumento da utilização dos métodos de imagem. A maioria são neoplasias mucinosas quísticas, consideradas lesões precursoras do adenocarcinoma do pâncreas (PDAC), e constituem uma excelente oportunidade para diagnóstico e tratamento precoces. O dilema na prática clínica, é distinguir os quistos pré-malignos de alto risco e malignos, que requerem tratamento cirúrgico, dos quistos benignos ou pré-malignos de baixo risco, que não devem ser "sobre-tratados" e podem nem requerer vigilância.

Atualmente é difícil diferenciar os quistos mucinosos pré-malignos dos quistos benignos não mucinosos. As características imagiológicas e a análise do líquido de quisto pancreático (PCF) são centrais no diagnóstico, mas com a metodologia padrão atual, o diagnóstico diferencial permanece um desafio e o dilema do "sobre-tratamento" é frequente. Além disso, a progressão para malignidade dos quistos mucinosos permanece incerta, sendo necessários novos biomarcadores que possam superar a precisão diagnóstica da displasia como marcador de alto-risco.

O objetivo do presente trabalho é analisar de forma abrangente biomarcadores e abordagens diagnósticas de PCL por Ecoendoscopia com punção (EUS-FNA), numa coorte de doentes com quistos predominantemente de baixo risco sob vigilância imagiológica. Estas lesões são muito mais frequentes na prática clínica do que as lesões malignas e contrasta com as séries publicadas que incluem predominantemente coortes de PCL de alto risco operadas, o que por si só constitui um viés de seleção. A análise de PCF realizada nesta coorte inclui estudos de genómica (mutações no DNA), epigenómica (análise de metilação), metabolómica (glicose) e proteómica (CEA, cromogranina A, NSE), incluindo potenciais biomarcadores para diagnóstico de quistos mucinosos que beneficiam de vigilância e para avaliação de malignidade que pressupõe ressecção cirúrgica. No final tentámos melhorar o organograma diagnóstico das PCL baseado nas *guidelines* atuais.

Começámos por abordar o papel dos marcadores moleculares, nomeadamente as mutações do *KRAS* e *GNAS* no diagnóstico diferencial das PCL. Realizámos uma primeira meta-análise comparando o CEA e a citologia com as mutações do *KRAS* para diagnóstico dos quistos pancreáticos. O CEA foi o melhor teste para os quistos clinicamente significativos (AUC=0.69), e a citologia teve a melhor performance nos quistos malignos (AUC=0.78), enquanto as mutações do *KRAS* falharam diagnósticos de quistos significativos e malignos com uma AUC de 0.53 e de 0.56, respetivamente. As limitações dos marcadores moleculares no PCF verificaram-se mesmo considerando apenas a avaliação com sequenciação de nova geração

(NGS) numa coorte de doentes cirúrgicos. Concluímos que, na atualidade, não há benefício da análise molecular para diagnóstico de PCL de alto risco ou malignos.

Numa segunda meta-análise, comparámos a precisão diagnóstica da análise molecular *versus* a biópsia com micropinça (MFB) no diagnóstico de PCL referenciadas para cirurgia. As duas abordagens revelaram-se idênticas no diagnóstico de quistos benignos, mas a análise molecular foi superior no diagnóstico tanto de quistos mucinosos de baixo risco como de alto risco (AUC de 0.95 e 0.92, respetivamente). No entanto, a rentabilidade diagnóstica foi mais elevada na MFB (0.73 vs 0.54). Embora os nossos resultados salientem o valor diagnóstico tanto da análise molecular como da MFB, concluímos que ambos os testes ainda requerem validação e não devem ser recomendados como a primeira linha na clínica.

Para além da meta-análise, realizámos um estudo retrospetivo para avaliar o valor adicional das mutações do *KRAS* e do *GNAS* em PCF de 52 amostras congeladas, das quais 21 pertencentes a lesões mucinosas (14 de baixo risco e 7 malignas). Embora as mutações do *KRAS* tenham sido detetadas predominantemente em quistos mucinosos e malignos, o nível de CEA nos quistos mucinosos de baixo risco e as características de imagem associadas à citologia nos quistos mucinosos/malignos de alto risco foram mais discriminatórios do que os marcadores moleculares. Assim, concluímos que em comparação com testes convencionais - CEA e citologia - não existe valor adicional das mutações do *KRAS* ou do *GNAS* no diagnóstico diferencial das PCL. Além disso, há outras desvantagens dos marcadores moleculares, incluindo custos significativos e complexidade técnica que dificultam a sua implementação na prática clínica.

Avançando da genómica para a metabolómica, explorámos o valor da avaliação do nível de glicose em PCF em comparação com o CEA, que é o marcador mais utilizado para distinguir lesões mucinosas de não mucinosas. Foram incluídos 82 doentes com lesões benignas, inflamatórias, pré-malignas e malignas. Para diagnóstico de quistos mucinosos, um valor de CEA >192 ng/ml apresentava uma AUC de 0.84, enquanto que para um valor de glicose <50 mg/dl, a AUC era de 0.86, sendo a sensibilidade da glicose (89%), superior à do CEA (72%). Concluímos que a glicose avaliada por um glicosímetro é mais precisa que o CEA para o diagnóstico de quistos mucinosos. Além da maior precisão diagnóstica, a glicose em PCF avaliada "in loco" com um glicosímetro, é fácil, imediata e requer uma quantidade mínima de PCF.

No estudo seguinte, o nosso objetivo foi avaliar se uma segunda EUS-FNA realizada em lesões selecionadas de alto risco incluídas num programa de vigilância alterou o diagnóstico ou a decisão terapêutica de quistos pancreáticos. Comparámos o resultado de 105 doentes com uma única EUS-FNA com o de 23 doentes que fizeram uma segunda EUS-FNA. O tempo médio entre as duas EUS-FNAs foi de 38 meses. 4/23 doentes foram referenciados para cirurgia após a segunda EUS-FNA, 2 dos quais com tumores neuroendócrinos (NET) quísticos. Concluímos que a repetição de EUS-FNA em lesões selecionadas, mesmo sem "*worrisome features*", pode ser recomendada, pois alterou o tratamento realizado para cirurgia em aproximadamente 20% dos doentes, em especial de NET quísticos.

Após estes resultados, explorámos o papel da EUS-FNA em pequenas PCL (<3 cm), pois as *guidelines* atuais apenas recomendam a realização de punção em PCL maiores que 3 cm. Foram incluídos 115 pacientes com PCLs <3 cm submetidos a EUS-FNA. 19/115 foram operados e 15 correspondiam a lesões malignas ou pré-malignas. Os quatro doentes restantes apresentavam lesões benignas. Concluímos que a realização de EUS-FNA em lesões com <3 cm pode melhorar o resultado e o custo-efetividade dos programas de vigilância, permitindo confirmar malignidade em 2 de 5 lesões ressecadas, e simultaneamente diagnosticar quistos benignos que podem ser libertados desses programas.

Os NET quísticos são considerados PCL de alto risco, que por vezes são sub-diagnosticados ou colocados em segundo plano nos algoritmos de diagnóstico atuais. O nosso objetivo foi avaliar o valor dos níveis de cromogranina A (CroA) e de enolase específica neuronal (NSE) em PCF para o diagnóstico de NET quísticos pancreáticos. Dezasseis doentes foram incluídos, dos quais 4 com NET quísticos. Os níveis de CroA e NSE foram mais elevados nos NET quísticos, com uma AUC de 0.94 para a CroA e 1 para a NSE. Apesar do baixo número de pacientes incluídos neste estudo piloto, concluímos que esses são biomarcadores promissores para identificar os NET quísticos pancreáticas.

Por fim, avaliámos alterações epigenéticas para o diagnóstico de quistos malignos. As alterações de metilação em 5 regiões diferencialmente metiladas (DMRs) do *locus GNAS* foram avaliadas para compreender se podem contribuir para a progressão maligna de PCL. Foram estudadas 52 amostras de PCF previamente caracterizadas com mutações do *KRAS* e *GNAS*. Observámos que as alterações na metilação do *locus GNAS* se associaram de forma significativa à ocorrência de malignidade - 6/8 quistos malignos e apenas 2/20 quistos benignos. Este é o primeiro estudo a identificar alterações de metilação no *locus GNAS*, com melhoria do diagnóstico de PCL malignas.

Como o desempenho de qualquer marcador isolado é imperfeito e a combinação de dados clínicos, morfológicos, bioquímicos e citológicos melhoram o diagnóstico, terminamos este trabalho propondo um organograma de diagnóstico aperfeiçoado que incorpora os resultados descritos nesta tese.

Com a evolução de novos biomarcadores em PCF, surgem questões de precisão, efeitos na orientação do doente, valor de um biomarcador individual *versus* de um painel de biomarcadores, e ordem da sua obtenção para dar o melhor suporte às decisões clínicas. Será necessário progredir com ensaios colaborativos de validação em larga escala e integração de novas estratégias, de forma a personalizar o risco de malignidade dos quistos pancreáticos em cada doente individual.

Abstract

Pancreatic cystic lesions (PCLs) are increasing incidental findings due to increased ageing of the population and widespread use of imaging. The main problem in clinical practice has to do with distinguishing the high-risk premalignant and malignant cysts that require surgical treatment from the benign or low-grade dysplastic cysts, which should not be over-treated and might not even require surveillance. The goal of the present work is to perform a comprehensive analysis of biomarkers and diagnostic approaches by Endoscopic Ultrasound with Fine-needle Aspiration (EUS-FNA), in a cohort of patients harboring mostly low-risk cysts under surveillance, which are far more frequent in clinical practice.

The PCF analysis performed in this cohort includes studies of genomics (DNA mutations), epigenomics (methylation analysis), metabolomics (glucose), and proteomics (CEA, chromogranin A, NSE), with putative biomarkers encompassing the diagnosis of mucinous and malignant cysts, that require surveillance and surgical resection, respectively.

We performed a first meta-analysis comparing current diagnostic methods - CEA and cytology - with *KRAS* mutations for the diagnosis of mucinous cysts. CEA was the best test for clinically significant cysts (AUC=0.69), cytology performed better in malignant cysts (AUC=0.78), surpassing *KRAS* mutations (AUC=0.53 and AUC=0.56, respectively). In a second meta-analysis we compared the accuracy of molecular analysis *versus* micro forceps biopsy (MFB) in the diagnosis of PCLs. The two approaches were identical for diagnosing benign cysts, but molecular analysis was superior for diagnosing both low and high-risk mucinous cysts.

In addition to these two meta-analyses, we performed a retrospective study evaluating the added value of *KRAS* and *GNAS* mutations in PCF of 52 frozen PCF samples. We conclude that, as compared with conventional tests, these had no added value in the differential diagnosis of PCLs. In another publication, we compared glucose level in PCF with CEA in 82 patients. For mucinous cyst diagnosis, a CEA >192 ng/ml showed an AUC of 0.84 while glucose <50 mg/dl revealed an AUC of 0.86. Besides its higher accuracy, PCF glucose evaluated "on site" with a glucometer is easy, immediate, and requires a minimal amount of PCF.

In the next study we sought to determine whether a second EUS-FNA changed the diagnosis or management of pancreatic cysts. We compared the outcome of 105 patients with a single EUS-FNA with that of 23 patients who had a second EUS-FNA. EUS-FNA may be recommended, as it changed management toward surgery in approximately 20% of the patients, particularly with diagnosis of cystic NETs. Following these results, we explored the role of EUS-FNA in small PCLs (<3 cm) in 115 patients with PCLs <3 cm who underwent EUS-FNA. 19/115 were submitted to surgery with 15 malignant or pre-

malignant lesions and the remaining 4 were benign lesions. We conclude that EUS-FNA in lesions <3 cm may improve outcome and cost-effectiveness of surveillance programs, as it confirmed malignancy in 2 out of 5 resected lesions, while it also diagnosed benign cysts who could be released from these programs. In a pilot study with 16 patients, including 4 cystic NETs we aimed at assessing the value of Chromogranin A (CroA) and neuron-specific enolase (NSE) levels in PCF. CroA and NSE levels were higher in cystic NETs with an AUC of 0.94 for CroA and 1 for NSE. These are promising biomarkers to identify pancreatic cystic NETs.

Finally, we studied epigenetic changes in the diagnosis of malignant cysts. Methylation changes of *GNAS locus* were evaluated to understand whether they may contribute to malignant progression of PCLs. Fifty-two samples of PCF were studied. We observed that *GNAS locus* methylation changes were significantly associated with malignancy. This is the first study to identify methylation changes in the *GNAS locus* improving diagnosis of malignant PCLs.

We end this work proposing a revised diagnostic organogram of PCLs established by current guidelines, that incorporates the results obtained in this dissertation's research.

Keywords

Pancreatic cysts; Endoscopic Ultrasound with fine needle aspiration (EUS-FNA); Pancreatic cystic fluid; CEA; Cytology; Biomarker

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List of Acronyms

Acronym	Description
ACC	Acinar cell carcinoma
ACG	American College of Gastroenterology
ADC	Adenocarcinoma
AGA	American Gastroenterology Association
ATRX	chromatin remodeler
AUC	Area under the curve
BD	Branch duct
BD-IPMN	Branch duct-IPMN
CEA	Carcinoembryonic antigen
CI	Confidence Interval
CroA	Chromogranin A
cfDNA	Cell free DNA
ctDNA	Circulating tumoral DNA
CT	Computed tomography scans
CTNNB1	Catenin beta 1
DAXX	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DMF	Digital Microfluidics
DMR	Differential methylated regions
DNA	Deoxyribonucleic acid
EU	European
EUS	Endoscopic ultrasound
EUS-FNA	Endoscopic ultrasound with fine needle aspiration
FAP	Familial adenomatous polyposis
FNA	Fine needle aspiration
GNAS	Guanine nucleotide binding protein, alpha stimulating
GRUBI	Grupo de Revisões Sistemáticas da Universidade da Beira Interior
HGD	High-grade dysplasia
IAP	International Association of Pancreatology
IPMN	Intraductal Papillary Mucinous Neoplasm
IPMN-CC	IPMN concomitant carcinoma
IPMN-DC	IPMN derived carcinoma
IPOLFG, EPE	Instituto Português de Oncologia de Lisboa de Francisco Gentil, EPE
KRAS	Kirsten rat sarcoma viral oncogene homolog
LGD	Low-grade dysplasia
LOH	Loss of heterozigoty
MA	Molecular analysis
mTOR	Mechanistic target of rapamycin kinase
McAS	McCune Albright Syndrome
MCN	Mucinous Cystic Neoplasm

MD	Main Duct
MD-IPMN	Main-duct IPMN
MEN1	Multiple endocrine neoplasia type 1
MFB	Micro forceps biopsy
µl	Microliter
MPD	Main pancreatic duct
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MT-IPMN	Mixed type-IPMN
MUC	Mucin
MUSC	Medical University of South Carolina
n-CLE	Needle-based confocal laser endomicroscopy
NET	Neuroendocrine tumor
NGS	Next generation sequencing
NLR	neutrophil-to-lymphocyte ratio
NOD	New onset diabetes
NPV	Negative predictive value
NSE	Neuron-specific enolase
PanIN	Pancreatic intraepithelial neoplasia
PCF	Pancreatic cystic fluid
PCL	Pancreatic cystic lesion
PDAC	Pancreatic ductal adenocarcinoma
PET	Positron emission tomography
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PJS	Peutz-Jeghers Syndrome
PPV	Positive predictive value
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
SCA	Serous cystadenoma
SE	Standard error
SPPN	Solid pseudopapillary neoplasm
sROC	Summary Reciever Operating Characteristic
TP53	Tumor Protein 53
UPD	Uniparental disomy
USPSTF	United States Preventive Services Taskforce
WF	Worrisome features

Chapter I.

Introduction

1. Pancreatic Cystic Lesions

Pancreatic cystic lesions (PCLs) are predominantly incidental findings in patients with non-specific abdominal pain, with a described prevalence around 2.5%.[2,3] The majority of pancreatic cysts are mucinous (58%) with inherent malignant potential.[4]

This pancreatic cyst "epidemic" is probably related to the generalized increase in use of multidetector computed tomography scans (CT) and magnetic resonance imaging (MRI), in progressively older patients. Endoscopic Ultrasound (EUS) is important for additional imagiological evaluation, but imaging *per se* is inaccurate to identify the exact nature of PCLs.

Therefore, most incidental PCLs require surveillance or additional evaluation, depending on cystic features and whether the patient is fit for surgery. Unfortunately, and despite all recent achievements, with current clinical and radiological evaluation, including EUS or not, the pre-operative diagnosis of incidental PCLs is still inaccurate in over a third of patients and 5% of the resected cysts may not even be neoplastic.[5]

1.1. Types of Pancreatic Cystic Lesions

PCLs are morphologically and genetically heterogeneous, ranging from benign to malignant lesions, with extremely poor clinical outcome. The wide spectrum of diagnosis encompasses benign/inflammatory lesions [e.g. serous cystadenomas (SCAs), pseudocysts], pre-malignant [intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs)], and malignant cysts [cystic adenocarcinomas (ADCs), cystic neuroendocrine tumors (NETs), acinar cell carcinomas (ACCs), etc.][6].

The clinical and morphological characteristics of PCLs are summarized in Table 1.

Table 1. Typical clinical and imaging features of most common pancreatic cysts.

Reproduced with permission from Tanaka M et al. (2012) International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. Pancreatology. doi:10.1016/j.pan.2012.04.004.[7]

Characteristic	MCN	BD-IPMN	SCN	Pseudocyst
Sex (% female)	>95%	~55%	~70%	<25%
Age (decade)	4th, 5th	6th, 7th	6th, 7th	4th, 5th
Asymptomatic	~50%	Mostly when small	~50%	Nearly zero
Location (% body/tail)	95%	30%	50%	65%
Common capsule	Yes	No	Yes	N/A
Calcification	Rare, curvilinear in the cyst wall	No	30–40%, central	No
Gross appearance	Orange-like	Grape-like	Spongy or honeycomb-like	Variable
Multifocality	No	Yes	No	Rare
Internal structure	Cysts in cyst	Cyst by cyst	Microcystic and/or macrocystic	Unilocular
Main pancreatic duct communication	Infrequent	Yes (though not always demonstrable)	No	Common
Main pancreatic duct	Normal or deviated	Normal, or dilated to >5 mm, suggesting combined type	Normal or deviated	Normal or irregularly dilated, may contain stones

Abbreviations: MCN, mucinous cystic neoplasm; BD-IPMN, branch duct intraductal papillary mucinous neoplasm; SCN, serous cystic neoplasm; N/A, not applicable.

The differential diagnosis of PCLs is difficult but crucial for clinical decision and patient management, with surgery required for high-risk and malignant cysts, surveillance recommended for premalignant lesions, and no necessary follow-up for benign cysts. Figure 1 displays features of different PCLs.

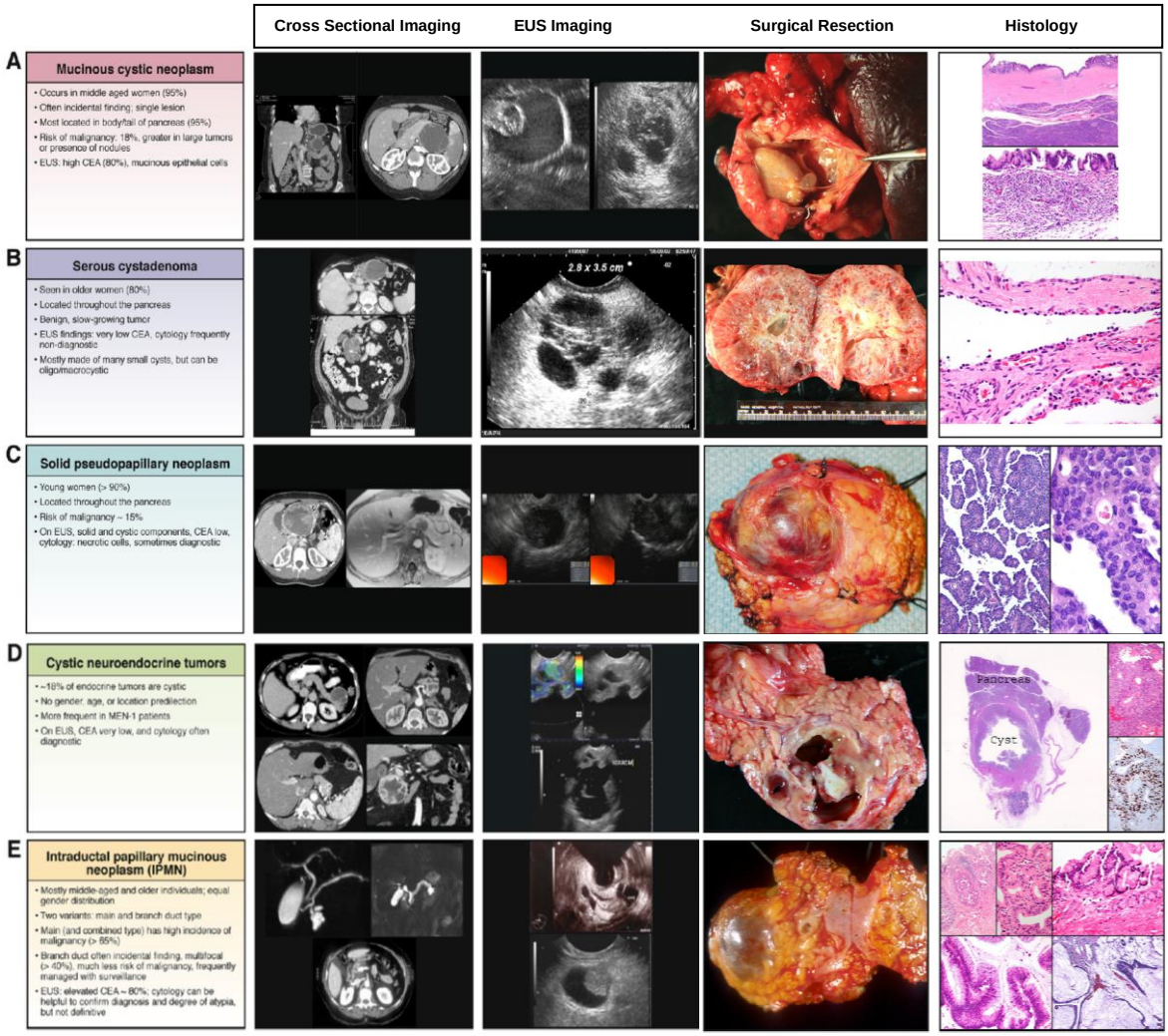


Figure 1. Imaging and histological features of the most common pancreatic cysts.
Reproduced with permission from Farrell JJ et al. (2013) Pancreatic Cystic Neoplasms: Management and Unanswered Questions. Gastroenterology. doi: 10.1053/j.gastro.2013.01.073.[8]

A diagnosis of a rare cyst with distinct and variable biological behavior should be considered as well, as it corresponds to 10% of all PCLs. These lesions include solid pseudopapillary neoplasms (SPPNs), cystic acinar cell neoplasms and cystic degeneration of solid pancreatic tumors, e.g. cystic ADCs, cystic NETs, and lymphangiomas, among others[9], as outlined in Table 2.

Table 2. Types of rare pancreatic cystic lesions.

Reproduced with permission from Sakorafas GH et al. (2012) Primary pancreatic cystic neoplasms of the pancreas revisited. Part IV: Rare cystic neoplasms. Surgical Oncology. doi:10.1016/j.suronc.2011.06.007.[9]

Rare primary pancreatic cystic neoplasms.
Solid pseudopapillary neoplasms
Cystic neuroendocrine neoplasms of the pancreas
Cystic acinar cell neoplasms
Cystic variants/degeneration of solid pancreatic neoplasms
Intraductal tubular neoplasms
Angiomatous neoplasms of the pancreas
Cystic lymphangioma
Cavernous hemagiomas
Pancreatoblastoma (cystic variant)
Lymphoepithelial cysts
Other rare cystic neoplasms
Cystic choriocarcinomas
Mature cystic teratoma
Pancreatic cystic hamartoma
Cystic neoplasms of mesenchymal origin

1.2. Imaging of Pancreatic Cystic Lesions

Imaging *per se* lacks accuracy for differential diagnosis of PCLs because there are no clear pathognomonic features of each cyst type, although some findings may suggest a particular diagnosis.

The imaging of PCLs reflects their histologic architecture. SCAs wall lining is composed of a glycogen-rich cuboidal epithelium whereas mucinous cysts have a mucin containing columnar epithelium, with MCNs differing from IPMNs because they have a characteristic ovarian-type stroma. IPMNs arise from the pancreatic ducts, and the mucin-producing epithelium can progress from low-grade dysplasia (LGD) to high-grade dysplasia (HGD) and eventually invasive carcinoma.[10] In SPPNs, the solid component is composed of pseudopapillae with a fibrovascular stalk forming pseudorosettes.[9] Cystic NETs contain debris and are lined by a ragged cuff or well-preserved neoplastic endocrine cells, while acinar cell neoplasms present layers of neoplastic acinar cells, sometimes forming minute lamina within the epithelial lining.[9]

Translating histology into imaging, SCAs are usually multilocular cysts with thin septae and may present a central scarring or calcification. However, 20% of SCAs may have a dominant macrocyst or even a solid component appearing identical to mucinous cysts. MCNs are almost exclusively located in the tail of the pancreas, are usually unilocular or have a small number of discrete compartments, and almost never communicate with the pancreatic duct. Rarely, they may present a peripheral egg-shell calcification, predictive of cancer. IPMNs can be subclassified as branch-duct (BD-IPMNs) if they correspond to dilated side-branch(es), as main duct (MD-IPMNs) if the dilatation occurs in the Wirsung, and mixed type (MT-IPMNs) if presenting simultaneous dilatation of the main pancreatic duct (MPD) and side-branches.

Rare pancreatic cysts typically present a solid component (e.g. SPPN), or a thick-wall (e.g. cystic NETs) or may be purely cystic (e.g. lymphangiomas), but atypical presentations are frequent and there are no pathognomonic diagnostic findings.[9]

The current limitations of imaging in the differential diagnosis of PCLs are significant, due to technical limitations, small size, identical architectural patterns shared by different histological cyst types, and atypical presentations.

1.3. Pancreatic Cyst Fluid Analysis

For additional evaluation, endoscopic ultrasound with fine-needle aspiration (EUS-FNA) plays a pivotal role in the evaluation of pancreatic cysts, as illustrated in Figure 2, allowing sampling and analysis of pancreatic cyst fluid (PCF) that adds crucial diagnostic and prognostic information.

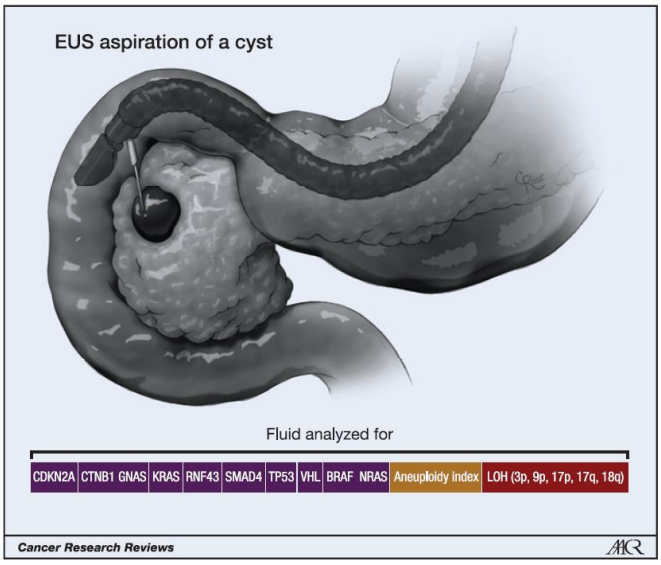


Figure 2. Pancreatic cystic fluid can be aspirated from a cyst at the time of EUS and be analyzed for CEA and cytology that are currently standard in clinics, as well as genetic markers, to distinguish among the various cystic lesions of the pancreas.
Reproduced with permission from Lennon AM et al. (2014) The Early Detection of Pancreatic Cancer: What Will It Take to Diagnose and Treat Curable Pancreatic Neoplasia? Cancer Research doi: 10.1158/0008-5472.CAN-14-0734.[11]

PCF is particularly relevant to distinguish between benign neoplasms, such as macrocystic SCAs, from pre-malignant lesions like BD-IPMNs and MCNs. Besides PCF, EUS-FNA with recently developed micro forceps biopsy (MFB), allows the acquisition of solid material for histological characterization, namely from the epithelium lining the cyst wall, or from the septa and mural nodules.

Standard analysis of PCF includes cytology, determination of CEA and amylase levels, although additional evaluation of viscosity, extracellular mucin, other tumor markers, and molecular analysis is also possible.

PCF cytology is very specific for malignancy, although paucicellular samples are common, reducing its sensitivity. As few viable cells shed from the cyst wall lining and patchiness of epithelial architecture is a frequent feature, obtaining a definitive cytological diagnosis is often problematic.

PCF fills the cystic space and contacts with the epithelium of the cystic wall, and its analysis became established as the best diagnostic tool for PCLs, since the publication of the cooperative pancreatic cyst study group in 2004. This study described 80% accuracy of CEA level in PCF using a cutoff value of 192 ng/mL for diagnosis of mucinous cysts.[12] It was found that the accuracy of CEA was greater than EUS imaging, cytology, or other PCF tumor markers including CA 72-4, CA 125, CA 19-9, and CA 15-3 for identifying IPMNs or MCNs.[12] Nevertheless, the sensitivity and specificity of CEA >192 ng/mL are limited to 73% and 84% respectively, with 25% of mucinous cysts presenting a CEA <192 ng/mL.[12]

More recently, a meta-analysis of 18 studies with 1438 patients found that CEA level in PCF had a sensitivity of 63% and a specificity of 88% for identifying IPMNs and MCNs.[13] In addition to only moderate sensitivity, some cysts are not amenable to fine needle aspiration (FNA) due to inaccessible location and the volume of PCF obtained to assess CEA level is often scant, particularly in small cysts or in cases in which the fluid is viscous. This was highlighted by a prospective study in which it was possible to obtain a cytopathologic diagnosis and a chemical analysis in only a third and half of cases, respectively, although EUS-guided FNA was technically feasible in the majority of patients with PCLs (87%).[14]

Some small, retrospective studies suggested that a high cyst fluid CEA is associated with HGD or invasive ADC in IPMNs, but this finding has not been confirmed in larger studies, including a large prospective study and a meta-analysis.[15,16]

In summary, CEA levels in PCF are of little help for the diagnosis of IPMNs with HGD or associated invasive ADC, while cytology is the best diagnostic tool, with 75% accuracy.[15] In addition, CEA is elevated in PCF of both IPMNs and MCNs, and is not useful in differentiating these cyst types. Chromogranin A (CroA) was studied in PCF for diagnosis of cystic NETs, with contradictory results.[17,18] Thus, despite a multidisciplinary approach, distinguishing different PCLs can be challenging.

Within the last decade, molecular techniques have emerged as a promising adjunct for evaluation of PCLs. Using deep sequencing technologies in minimal amounts of PCF, recurrent mutations characteristic of the major PCLs have been uncovered, with potential to improve the diagnosis and management of PCLs. In fact, despite the scant cellularity of PCF aspirates, DNA sheds from lysed or exfoliated cyst epithelial lining into the PCF and becomes available for molecular analysis.

Numerous studies have shown that DNA molecular analysis of aspirates obtained by EUS-FNA provide a better characterization of PCLs as compared to current methods used in clinics.[19,20,21,22,23,1,24,25,26] However, these studies have been largely retrospective, included mainly surgical specimens, and most lack adequate follow-up.

Next-generation sequencing (NGS) is the current standard for molecular analysis, given its increased sensitivity to detect smaller amounts of DNA and the ability to assay multiple genes simultaneously.[27] It reliably allows the analysis of PCF and peripheral blood,

and is an attractive option to increase the diagnostic accuracy and to enable the risk stratification of PCLs.[28]

KRAS mutation is extremely specific for mucinous cyst types (IPMNs and MCNs) while *GNAS* mutation is specific for IPMNs. Additional genetic mutations in *TP53*, *PIK3CA*, and *PTEN* have been associated with advanced neoplasia in mucinous cysts with a high sensitivity and specificity of 91% and 97%, respectively.[29,30]

Concerning rare cysts, almost 95% of SPPNs present activating mutations in the ß-catenin gene (exon 3 of *CTNNB1*), NETs present mutations in *MEN1*, *DAXX*, *ATRX* and genes of the mTOR pathway and ACCs present significant genomic instability and abundant mutations, even more numerous than pancreatic ductal adenocarcinoma (PDAC).[31] Genetic mutations characteristic of different PCLs are displayed in Table 3.

Table 3. Frequently targeted genes in pancreatic neoplasms.
Reproduced with permission from Rishi A et al. (2015) Pathological and molecular evaluation of pancreatic neoplasms. Seminars in Oncology. doi: 10.1053/j.seminoncol.2014.12.004.[31]

Pancreatic Neoplasm	Targeted Gene	Alteration Prevalence	Altered Gene Function
PDCA	*KRAS*	90%	Cell cycle activation (MAPK and PIK3CA pathway)
	P16/CDKN2A	95%	CDK4 and CDK6 inhibition
	TP53	75%	Cellular stress response
	SMAD4	55%	Loss of TGF-β induced tumor suppression
IPMN	*KRAS*	80%	Cell cycle activation (MAPK and PIK3CA pathway)
	P16/CDKN2A	Present only in high-grade dysplasia and carcinoma	CDK4 and CDK6 inhibition
	TP53		Cellular stress response
	SMAD4		Loss of TGF-β induced tumor suppression
	GNAS	60%–65% IPMN variants Intestinal: 100% Pancreatobiliary: 71% Gastric type: 51%	Uncontrolled growth signaling
	RNF43	75%	Wnt signaling regulation
MCN	*KRAS*	30%–80% Progressive increase with dysplasia grade	Cell cycle activation
	RNF43	40%	Wnt signaling regulation
	P16/CDK2NA, TP53, SMAD4	Only in high-grade tumors	
SPN	*CTNNB1*	95%	Wnt/β-catenin signaling pathway activation
PanNET	*MEN1*	45%	
	DAXX and ATRX	45%	Chromatin remodeling
	PIK3CA, PTEN and *TSC2*	14%	mTOR pathway
	VHL	25%	HIF-1α pathway
ACC	APC–β-catenin	25%	Cell signaling and adhesion
	KRAS	Rare	Cell cycle activation
	TP53	Rare	Cellular stress response
	Fanconi anemia pathway genes	45%	DNA repair mechanism
	CTNNB1	5%	Cell signaling and adhesion
PB	APC–β-catenin	86%	Cell signaling and adhesion
	CTNNB1	55%	Cell signaling and adhesion

Abbreviations: PDCA, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary and mucinous neoplasm; MCN, mucinous cystic neoplasm; SPN, solid and pseudopapillary neoplasm; PanNET, pancreatic neuroendocrine tumor; ACC, acinar cell carcinoma; PB, pancreatoblastoma.

These studies have limitations, and large multicenter validation studies are still missing. Moreover, the significant costs, logistic difficulties in collecting and preserving material for future molecular analysis in busy general hospitals, and the technical complexity of the test, make its generalized use difficult in clinical practice.[32] Additionally, there is a need for more trials to confirm the clinical relevance of molecular analysis in patient outcomes[33,34], such as early cancer diagnosis, number of surgeries of benign lesions avoided, and prognostic value in the numerous cysts that require periodic surveillance.

Currently, the integration of molecular analysis in routine clinical practice is still a matter of debate and, with the evidence currently available, is recommended only as a second line testing, with the diagnosis and prognosis of PCLs in clinical practice relying on a combination of clinical data, imaging features, and EUS-FNA for PCF analysis including CEA and cytology.[1,35,29]

Besides genomics, several promising biomarkers have been studied in small retrospective series, e.g. proteins, microRNAs, mucin profiling, and monoclonal antibody, but additional validation studies are still required.[36]

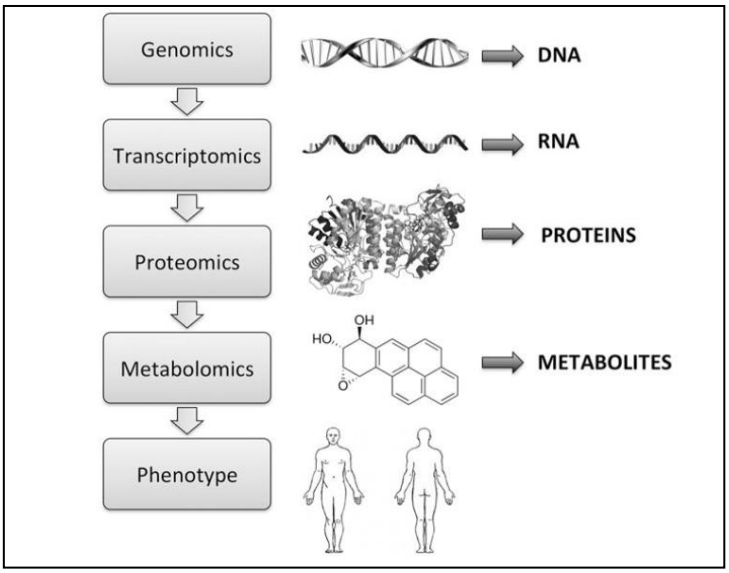


Figure 3. The "omnics" cascade in the diagnostic evaluation of PCLs. It includes genomics, transcriptomics, proteomics, and metabolomics. Metabolomics, the newest member, encompasses the high-throughput identification and quantification of small metabolites (e.g. glucose) and their interactions within biological networks.
Reproduced with permission from Turkoglu O, et al. (2016) Metabolomics of biomarker discovery in ovarian cancer: a systematic review of the current literature. Metabolomics. doi: https://doi.org/10.1007/s11306-016-0990-0[37]

Last but not least, only PCLs presenting diagnostic uncertainty, in which PCF results may influence patient management, should be evaluated by EUS-FNA, as adverse events are reported in 2.7% of patients, particularly abdominal pain, pancreatitis, intra-cystic bleeding, and cyst infection, with current guidelines recommending routine antibiotic prophylaxis, despite the conflicting evidence.[38,39]

1.4. EUS-guided Assessment of the Cystic Wall

The difficulties in the diagnosis of PCLs led to the development of new EUS-guided technologies, although with limited results. Needle-based confocal laser endomicroscopy (n-CLE) enables real-time optical biopsies, providing *in vivo* histopathologic assessment during EUS-FNA using a 19-gauge needle.[40] Using n-CLE, SCAs demonstrate a typical superficial vascular network, whereas IPMNs exhibit papillary projections with an epithelial border and a vascular core.[41] MCNs present a gray band with a thin dark line, reflecting the epithelial lining of the cyst wall, with or without deep blood vessels, while pseudocysts are seen as a field of bright, gray and black particles, reproducing the inflammatory tissue lacking epithelial lining.[41] These studies, although promising, present some weaknesses, namely limited number of patients, poor interobserver agreement, and limited accuracy. Moreover, there are inherent restrictions to massive implementation of this technique in clinical practice related to expensive equipment, a long learning curve, and a minimum cyst size of 2 cm required for evaluation. Additional prospective studies with larger registries are awaited to clarify the real utility of these procedures.

The imperative clinical need for improvement in PCLs diagnosis led to the recent development of a through-the-needle miniature biopsy device for use during EUS-FNA[42,43]. The Moray micro forceps biopsy (MFB) device (US Endoscopy, Mentor, Ohio) is disposable and can pass through a standard 19-gauge needle that is already routinely used for EUS-FNA. It allows tissue sampling from the cyst wall, septa, or mural nodules, adding to standard PCF analysis the histological evaluation of the epithelial architecture and subepithelial stroma. Beyond the technical success and safety profile[44], the new device has shown to improve the diagnostic accuracy of specific cyst subtypes.[45,46] Another major advantage of histology over other new techniques for cyst diagnosis is that histologic analysis follows standard definitions and is already a routine in clinics. The major limitation of MFB is sampling error and denudation of epithelium of mucinous cyst, limiting its diagnostic accuracy. It is prudent to use MFB in combination with current methods of cyst evaluation, including EUS morphology, and PCF analysis for CEA and cytology, each with its limitations.

A recent retrospective study showed that the addition of MFB and/or n-CLE to standard cyst fluid chemistry and cytology resulted in a significantly higher rate of specific PCLs diagnostic classification, with major impact in clinical management decisions including need for continued surveillance or surgery.[47] The authors propose the addition of MFB and/or n-CLE to standard PCF analysis when performing EUS-FNA of PCLs, although prospective studies and cost analysis are warranted.

1.5. Strategy for Diagnosis and Clinical Management of Pancreatic Cystic Lesions

The natural history of PCLs is still unclear and malignant transformation, although rare, is a major concern. Currently, in patients with incidental PCLs without MPD dilation, the

presence of mural nodules and size over 3 cm are regarded as the best markers of malignancy. Mural nodules in the cyst wall suggest the presence of cancer or HGD[48], but it can also represent just a mucin aggregate. The latter is usually round, with smooth edges, anechoic center, and an echogenic rim in EUS imaging.

Contrast enhanced EUS can highlight the epithelial nature of a mural nodule while a vanishing nodule after standard FNA with cyst aspiration confirms its mucinous nature. Although size >3 cm is mentioned in current PCLs guidelines as a worrisome feature, it should prompt resection only if symptomatic or if additional features of malignancy are detected.

In summary, aspiration of cyst content, including PCF, mural nodules, septa, or cyst wall thickened areas is recommended for diagnosis and clinical management of PCLs, if imaging alone is not conclusive. Although mucinous cysts are the most common type, and usually require only surveillance, NETs and ADCs with cystic degeneration, as well as SPPNs and ACCs should undergo surgical treatment. In these lesions, EUS-FNA is able to establish the diagnosis pre-operatively and is particularly relevant to guide surgery, especially in small lesions with improved prognosis.

For clinical management of PCLs, assessment of cancer risk is determinant, as presented in Figure 4.

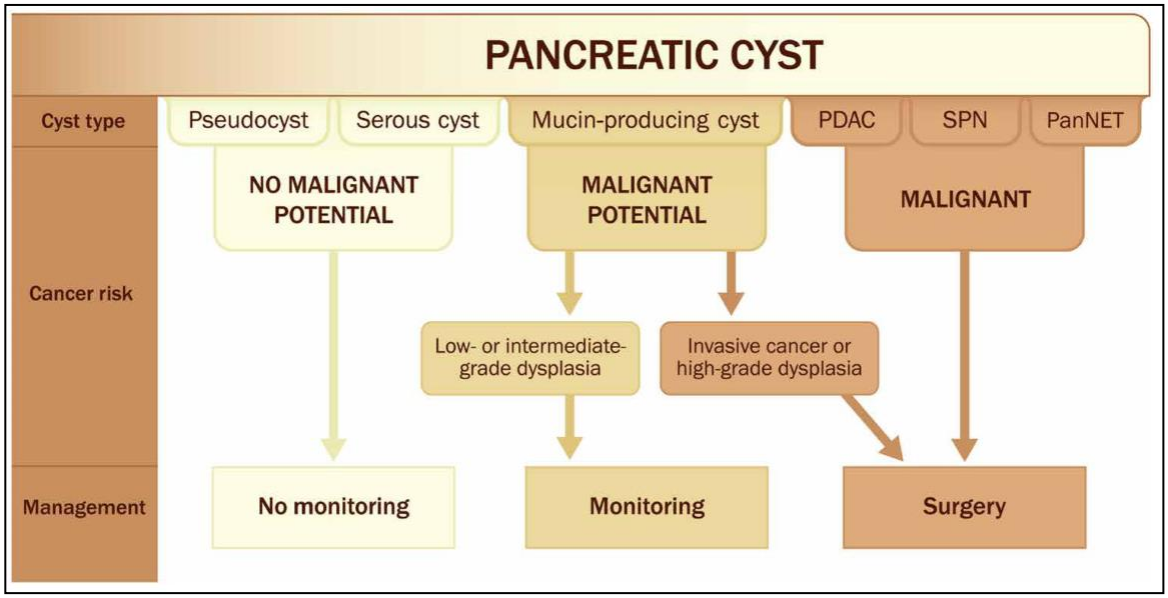


Figure 4. Clinical management of patients with pancreatic cysts. This figure shows how the type of pancreatic cyst dysplasia or associated invasiveness determines the risk of the cyst developing cancer, which in turn dictates clinical management. SCAs and pseudocysts have essentially no malignant potential and therefore require no monitoring. In contrast, cystic degeneration of a PDAC, NET, or SPPNs are, or have a high risk for becoming, malignant, and therefore should undergo surgical resection. IPMNs and MCNs are mucin-producing cysts. A small number of these harbor HGD or cancer and should be surgically resected, while the remaining mucin-producing cysts simply need surveillance.

Reproduced with permission from Springer S et al. (2019) A multimodality test to guide the management of patients with a pancreatic cyst. Sci Transl Med. doi: 10.1126/scitranslmed.aav4772.[49]

However, in most instances in clinical practice this algorithm is difficult to follow, as the differential diagnosis of PCLs using the current standard methodology is still a challenge

and we frequently face the dilemma of over treatment. Management recommendations have changed considerably over the last two decades, restraining surgery, as our understanding of the biology, frequency, and natural history of the different cyst types has improved. This evolution has resulted from the identification of clinical, radiological, and biological predictors of behavior.

With emerging technology and improvement of data management, a boost in diagnostic accuracy and individualized treatment of PCLs is expected and is urgent in the face of the current "cyst epidemic".

2. Relevance of Pancreatic Cystic Lesions

2.1. Early Diagnosis of Pancreatic Adenocarcinoma

Pancreatic cancer is currently the fourth leading cause of cancer death in the USA and is expected to be the second by the year 2030.[50,51] PDAC represents over 90% of all pancreatic malignancies, with the remaining being predominantly NETs.

The dismal five-year survival for PDAC, less than 10%, is related to late diagnosis, with the majority of patients presenting locally advanced or metastatic symptomatic disease (80-85%) and only a minority is eligible for surgical resection (15-20%).[52] On the contrary, it has been suggested that the 5-year survival rate for early PDAC (≤10 mm) may reach 80%.[53]

A major focus of research is the development of biomarkers and highly accurate imaging methods for earlier diagnosis of PDAC, increasing the proportion of surgical resections, with expected improvement in survival. Early detection of PDAC is not a goal for the general population as it is not helpful and may even be harmful.[54] The most recent recommendation statement of the United States Preventive Services Taskforce (USPSTF) presented in 2019 is against screening of PDAC in the general population[55,56], as the potential benefits of screening for PDAC in asymptomatic adults do not outweigh the potential harms. The potential harms include both false-positive results and the harm of treatment as there is still no evidence that screening for PDAC is effective in reducing mortality.

The low incidence of PDAC in the average risk population (12.9 per 100 000) leads to a reduced pretest probability that a positive test result represents a truly positive test. Thus, even an extremely specific screening test (specificity of 99%), would result in large numbers of false positives when applied to the general population, causing anxiety, superfluous imaging evaluation, and additional invasive tests. Perhaps even unnecessary surgery could occur in individuals with false positive test results. The potential overdiagnosis and overtreatment of PDAC is important and unacceptable due to significant mortality and morbidity related to pancreatic surgery.

An additional setback to overcome in early diagnosis of PDAC is that the multiple biomarker assays published so far (e.g. proteins, circulating DNA, microRNAs, methylated DNA, exosomes) were performed in cohorts of patients with symptomatic disease (a

diagnostic biomarker context) with scant data in the setting of longitudinal surveillance cohorts of asymptomatic individuals (a surveillance biomarker context).

To restrain overdiagnosis, early detection efforts of PDAC should focus in individuals at higher than average risk, who may benefit from surveillance. Nowadays there are well-defined risk-groups with well-quantified degrees of risk (greater than 5% of lifetime risk of pancreatic cancer or a 5-fold increase relative risk) and well-established precursor lesions. The next step is to determine how and how-often to conduct surveillance of at-risk individuals and which are the best modalities (biomarkers or imaging) for surveillance (Figure 5).

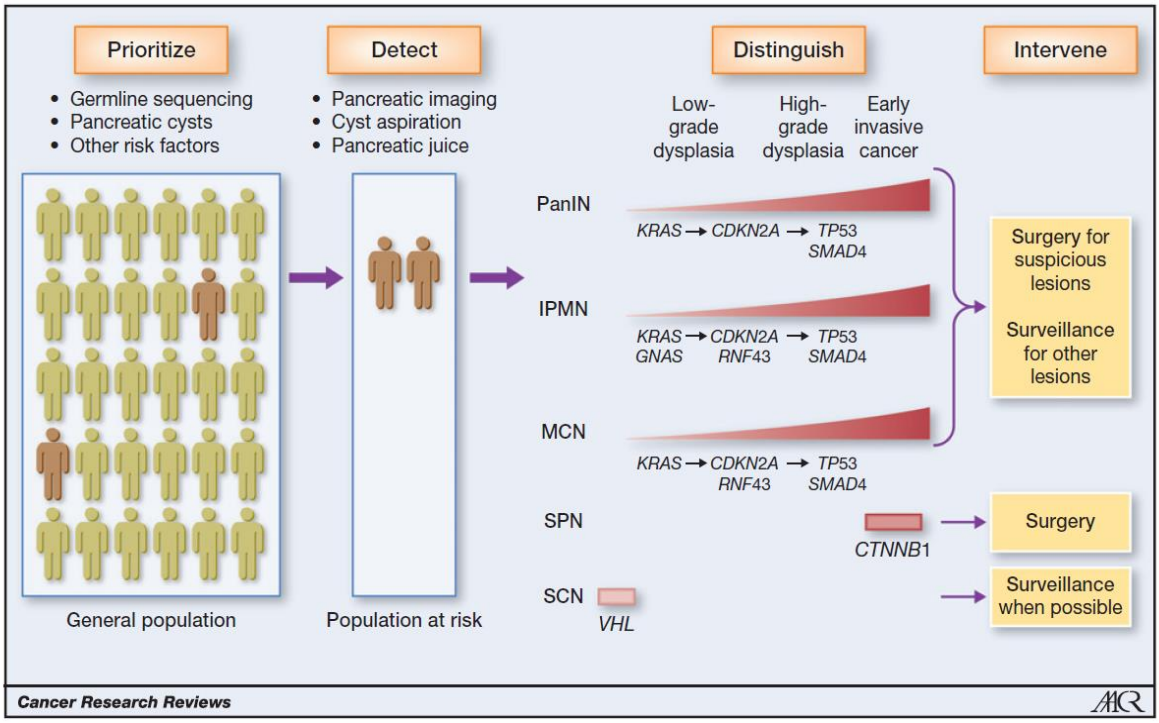


Figure 5. Steps required for the early detection of curable pancreatic neoplasia.
Individuals with an inherited genetic abnormality that increases their risk of developing PDAC, as well as individuals without a known predisposing gene mutation but who are predicted to be at increased risk based on their family history, are a first group to benefit from screening. Another way to increase the prevalence of a disease in a population is to select some individuals known to have a preexisting condition that predisposes to PDAC, as is the case of pancreatic cysts detected by CT and MRI. The screening of patients with multiple risk factors, such as elderly individuals with family history and a pancreatic cyst, would lead to even higher positive predictive values for any early screening test. Genetic markers studied in PCF have the potential to distinguish among the various cystic lesions of the pancreas and therefore could help distinguish harmless lesions and precursor lesions. SCAs are characterized by *VHL* gene alterations, SPPNs by *CTNN1* (β-catenin) gene mutations, IPMNs by *KRAS*, *GNAS*, *RNF43*, *TP53*, *p16/CDKN2A* and *SMAD4* gene mutations, and MCNs by *KRAS*, *RNF43*, *TP53*, *p16/CDKN2A*, and *SMAD4* gene mutations.
With these advances becoming a reality, we can easily envision that harmless cysts of the pancreas would be readily distinguishable from true precursor lesions in the near future.
Reproduced with permission from Lennon AM et al. (2014) The Early Detection of Pancreatic Cancer: What Will It Take to Diagnose and Treat Curable Pancreatic Neoplasia? Cancer Research doi: 10.1158/0008-5472.CAN-14-0734.[11]

The four major risk-groups defined for early diagnosis of pancreatic cancer are inherited (familial) pancreatic cancer, long standing history of pancreatitis, elderly patients with new-onset diabetes (NOD), not in the scope of this dissertation, and mucinous cysts.

Regarding the known precursors of PDAC, there are three different types of lesions, one microscopic and two macroscopic, which may be targetable for early diagnosis. The most common precursor is pancreatic intraepithelial neoplasia (PanIN), a microscopic lesion graded from low-grade (PanIN1 and PanIN2) to high-grade (PanIN3) dysplasia comprising epithelial proliferations in smaller pancreatic ducts. The other two precursors are mucinous cysts, presenting as macroscopic lesions, including the most frequent IPMNs, and MCNs.

IPMNs originate in the pancreatic ductal system, by definition are larger than 1 cm in size, can be graded from low to high-grade according to architectural and cytological atypia, and include four histological subtypes (intestinal, gastric, pancreato-biliar, and oncocytic) according to the phenotype of the epithelial lining.

MCNs do not involve the ductal system and have a pathognomonic ovarian-like stroma, even though having considerable genetic overlap with IPMNs.

For both types of mucinous cysts, *KRAS* mutations are a common initiator, but no *GNAS* mutations have been reported in MCNs[57], while they are frequent in IPMNs. Also, mutations in other suppressor genes such as *RNF43*, *SMAD4,* or *P53* occur later in MCNs.[57,58] Further clarification of the sequential genetic alterations that underlie the multistep tumorigenesis process in mucinous cysts may provide insights into pancreatic cancer biology and enable new strategies for early detection and optimized clinical care of patients with PDAC.[59]

Although high-quality data are lacking, the majority of cancers seem to originate from PanINs, with IPMNs an MCNs accounting for the initiation of 15-30% of cancers.[60] IPMNs, that originate from the pancreatic ductal system evolve from LGD to HGD to invasive carcinoma.[61,54] When subclassified as MD-IPMN, MT-IPMN, and BD-IPMN subtypes, these represent 15-21%, 22%-30%, and 41%-64% of the IPMNs, respectively. The comparison of average age of patients with non-invasive IPMNs and patients with IPMNs with invasive carcinoma suggest a three to five year-window between the detection of an IPMN and its progression to invasive cancer.

In summary, screening for pancreatic cancer in asymptomatic, average-risk individuals should not be recommended. Enrichment of screening population with individuals who carry increased risk of pancreatic cancer would alter screening in favor of surveillance in specific populations.[60,62,63,64] Recent studies have begun to provide support for treatment of screen-detected pancreatic cancer and precursor lesions, improving pancreatic cancer survival rates.[65] Further studies are needed to fully define the population who should be screened and the optimal strategy to improve outcomes and minimize harms resulting from surveillance in individuals at increased risk.

2.2. Epidemiological Risk Factors for Pancreatic Mucinous Neoplasms

There are several risk factors for developing IPMNs and IPMN-derived carcinomas (IPMN-DCs).

Advancing age is a major risk factor for the development of both lesions, with up to 10% of adult population[66] and 40% of individuals over the age of 80 years having a pancreatic cyst.[67] Overall, half of all IPMNs are BD-IPMNs, and age is a risk factor for both BD-IPMNs and BD-IPMN-DCs.[68] On the other hand, in a surgical series of MD-IPMNs, mean age of patients was 67 years old, identical for lesions harboring HGD and LGD.[69] Aging is the most important risk factor of pancreatic cancer. Age-related pathological changes play a key role in pancreatic carcinogenesis via the accumulation of gene mutations, epigenetic dysregulation, telomere dysfunction, and an altered stromal microenvironment.[70]

Diabetes is associated with IPMNs, malignant IPMNs, and IPMN concomitant carcinomas (IPMN-CCs). Among patients with BD-IPMNs, 10%-45% have a history of diabetes and diabetics have a higher incidence of BD-IPMNs.[71,72] A prior study by Capurso *et al.*[73] identified a strong association between insulin use and the risk of IPMNs. New-onset diabetes (NOD) or worsening in glycemic control in a known diabetic is also a predictor of IPMN-CC.[54] On the other hand, NOD is not associated with IPMN incidence in the absence of cancer, suggesting that IPMNs do not produce the same diabetogenic substances as PDAC. Emerging epidemiologic data demonstrate that individuals with NOD have up to an 8-fold greater risk of pancreatic cancer. Diabetes in this setting is distinct from type 1 and type 2 diabetes and is pancreatic in origin, which may result from a paraneoplastic process, with hyperglycemia being detected from 36 to 30 months before PDAC detection.[74]

Chronic pancreatitis increases the occurrence of both BD-IPMNs and BD-IPMN-DCs.[73] On one hand, BD-IPMNs can mimic retention cysts in the context of chronic pancreatitis. On the other, chronic pancreatitis may result from longstanding occlusion of the pancreatic duct due to mucin produced within the BD-IPMN itself.

Some genetic syndromes and a family history of PDAC may increase the risk of IPMNs. These have been reported in patients with Peutz-Jeghers syndrome (PJS), McCune Albright syndrome (McAS), and familial adenomatous polyposis (FAP).[75,76,77]

Patients with PJS have an elevated risk of malignancy, most commonly affecting the gastrointestinal tract, pancreas, breast, testis, and ovary. Germline and somatic mutations in the *STK11/LKB1* gene have been reported in a subset of PDAC. Sequencing analysis of the *STK11/LKB1* gene with loss of heterozygoty (LOH) in IPMNs revealed a germline mutation in one IPMN that occurred in a patient with PJS and a somatic mutation in 1 of 20 sporadic IPMNs, with no hypermethylation of the *STK11/LKB1* gene, suggesting that this gene may be involved in the pathogenesis of some IPMNs.[75]

McAS is a rare disorder characterized by fibrous dysplasia of bone, café-au-lait macules, and hyperfunctioning endocrinopathies, and is caused by somatic *GNAS* dominant-activating mutations, identical to those reported in IPMNs.[76] IPMNs have already been

described as a McAS–associated tumor, and are present in about 15% of patients. Further determination of the natural history and malignant potential of IPMNs in McAS is needed.

FAP is characterized by the development of hundreds to thousands of colorectal adenomas, and predictable colorectal cancer, caused by *APC* gene inactivation. Patients with FAP face other extracolonic lesions including benign (osteomas, odontomas, epidermoid cysts, desmoid tumors, congenital hypertrophy of the retinal pigment epithelium, fundic gland polyposis) and malignant (adenocarcinoma of the duodenum, thyroid, pancreas, biliary tract, stomach, and tumors of the liver or central nervous system) lesions. A case of IPMN in the pancreas was reported with genetic analysis showing both *APC* alleles inactivated in the IPMN, demonstrating that IPMN may be included as an extracolonic localization of FAP.[77]

Finally, in some studies it has been suggested that a history of PDAC in a first-degree relative increases the incidence of BD-IPMNs and BD-IPMN-DCs.[73] It is still unclear whether individuals with a family history of PDAC have more rapidly progressive IPMNs to malignancy.

In summary, there are several risk factors for developing IPMNs and IPMN-DCs, some also considered important for occurrence of PDAC. This finding supports the hypothesis that there is an overlap of factors influencing the carcinogenesis in these entities.

2.3. Diagnosis of Mucinous Cysts

The widespread use of advanced abdominal imaging in an ageing population has increased the detection of asymptomatic PCLs[78], including IPMNs and MCNs, precursors of PDAC.

The incidence of IPMN-DCs varies according to the morphological classification of IPMNs, with 11% to 80% of malignancy in MD-IPMNs and 20% to 65% in MT-IPMNs.[7,79] This high incidence of malignancy justifies the recommendation for surgical resection in both cyst types. BD-IPMNs are the most frequent type, have the most challenging differential diagnosis from other PCLs[54], and also present intrinsic malignant potential, expressed as 10% of non-invasive and 13% of invasive carcinomas in surgical specimens.[80] Because these statistics are based on surgical series, the malignant potential of IPMNs may be overestimated and further confirmation is required, particularly in BD-IPMNs, in which 80% of resections correspond to low-grade mucinous cysts.[10]

The key for early cancer detection in mucinous cysts is the accurate diagnosis of the most frequent asymptomatic BD-IPMNs and the identification of HGD and IPMN-DCs. Nevertheless, patients with BD-IPMNs also have increased risk of IPMN-CCs, with incidences varying from 1.1% to 11.2%[53], often diagnosed after 5 years of surveillance. The estimated incidence of PDAC in the age- and gender-matched control group is 0.045% per year.

IPMN-CCs occur more frequently in older individuals (>70 years old) and women, presenting benign gastric-type IPMNs without *GNAS* mutations and a family history of PDAC, including a single first-degree relative. IPMN-CCs are not considered by current guidelines,

but their occurrence probably justifies a full pancreatic evaluation strategy in surveillance of PCLs using EUS, as it is the best imaging technique to detect small solid lesions, and not stopping surveillance of stable PCLs even after 5 years, as recommended by AGA guidelines.[81]

Most pancreatic mucinous cysts are asymptomatic, with hypothetical diagnosis established with a high CEA level in PCF, although several pitfalls have been reported with this biomarker. First, the cut-off levels differ between laboratories[1,82,83], with limited reproducibility and difficulties in result interpretation. Second, a significant volume of PCF [at least 200 microliters (µl)] is required for CEA analysis, precluding its measurement in scant PCF samples. Finally, the currently used CEA level >192 ng/mL has limited diagnostic sensitivity, with considerable overlap in CEA levels between mucinous and non-mucinous cysts.[82]

Glucose, an easy and immediate "in-room" biomarker in PCF, appears to be an alternative to identify mucinous cysts, although limited evidence is so far available.[84,85,86,87]

To increase the diagnostic yield, genomic biomarkers in PCF have also been explored, particularly *KRAS* and *GNAS* mutations, but involve considerable complexity and costs.[36,88,89,58]

2.4. Detection of Progression in Mucinous Cysts

Currently, the timing and frequency of malignant progression in IPMNs remains unknown, leading to controversies in management.[90] This controversy is due to limitations in laboratorial, endoscopic, cytological, and imaging technologies that are still unable to reliably distinguish low risk IPMNs (presenting LGD and IGD) from those at high risk (HGD) and to assess the likelihood of progression to invasive cancer.

Currently, the most accurate factor associated with HGD in IPMNs is dilatation of the MPD on imaging, corresponding to MD-IPMNs that present a 50% to 60% chance of HGD or invasive ADC on resection.[91] The risk of pancreatic malignancy in BD-IPMNs is low but definite and concomitant dilatation of the MPD should prompt additional evaluation with EUS for cytological analysis whenever feasible.[92]

Most IPMNs arise from side duct branches, rarely progress to malignancy, and require only conservative surveillance. The malignancy rate is about 3.7% with an estimated annual incidence rate of 0.7% for BD-IPMNs under surveillance.[93] In a publication including 22% of high-risk BD-IPMNs on surgical pathology specimens, all of these lesions were larger than 3 cm, had mural nodules, or were symptomatic.[94] In another recent publication, malignancy developed in 8% of patients under surveillance[95], and in a large series, the 5-year incidence rate of pancreatic malignancy was 3.3%, reaching 15% at 15 years after diagnosis[96], with heterogeneous risk factor profiles between IPMN-DCs and IPMN-CCs. Although the size of BD-IPMNs and the diameter of the MPD were associated with IPMN-DCs, there was no association between cyst size and MPD diameter with IPMN-CCs.[96]

The scenario outlined justifies the generalized agreement of previous and current guidelines[7,97,79,98] in recommending resection for MD-IPMNs and surveillance for BD-IPMNs, with surgery indicated in the latter if "high-risk stigmata" are detected on imaging. Looking at the data from the opposite perspective, 40% of patients with MD-IPMNs and around 80% of patients BD-IPMNs will have low-risk disease at resection. Our current ability to identify high-risk disease in patients with IPMNs is limited and boosting this capacity would improve clinical care. More accurate biomarkers of HGD would allow a more rational treatment decision, avoiding morbid and life-threatening surgeries in low risk patients, and performing surgical resections in high-risk patients before invasive disease develops. Pancreatic surgery continues to be associated with a 2% to 4% risk of mortality[99] and an overall morbidity rate of 45%, even in referral centers with the largest surgical series.[100]

Recently, additional models have been studied in a preoperative setting and improved the diagnosis of high-risk lesions.[101,102] Their applicability must be confirmed in non-surgical cohorts, which present lower malignancy rates.

To predict the natural course of IPMNs it is necessary to understand the disease's biology and pathogenesis. The histopathological classification of IPMNs outlined in Figure 6[103] is important in progression and prognosis.

The most common IPMN subtypes are gastric and intestinal, seen in 60-70% and in 30-40% of patients, respectively. Gastric IPMNs are mostly of BD type (>95%), predominantly low-grade and have low risk of progression, although 5% are associated with invasive tubular carcinoma.

Intestinal IPMNs are characteristically MD-IPMNs and typically present HGD. Progression of this histological subtype is presumably an invasive colloid carcinoma which has better survival than invasive tubular carcinoma. Both intestinal IPMN and colloid carcinoma are associated with *GNAS* mutations.

Pancreatobiliary IPMNs can be BD-IPMNs or MD-IPMNs, are typically associated with HGD, and progress to a tubular-type carcinoma. Invasive tubular carcinoma in IPMN behaves identically to PDAC and confers a poor prognosis.[104]

Oncocytic sub-type is the least common, typically has HGD, and is generally presumed to have better prognosis than other IPMN sub-types of similar grade. It has no associated *KRAS* or *GNAS* mutations, and may be considered a different pathologic entity, distinct from gastric, pancreatobiliary, and intestinal IPMNs.[105]

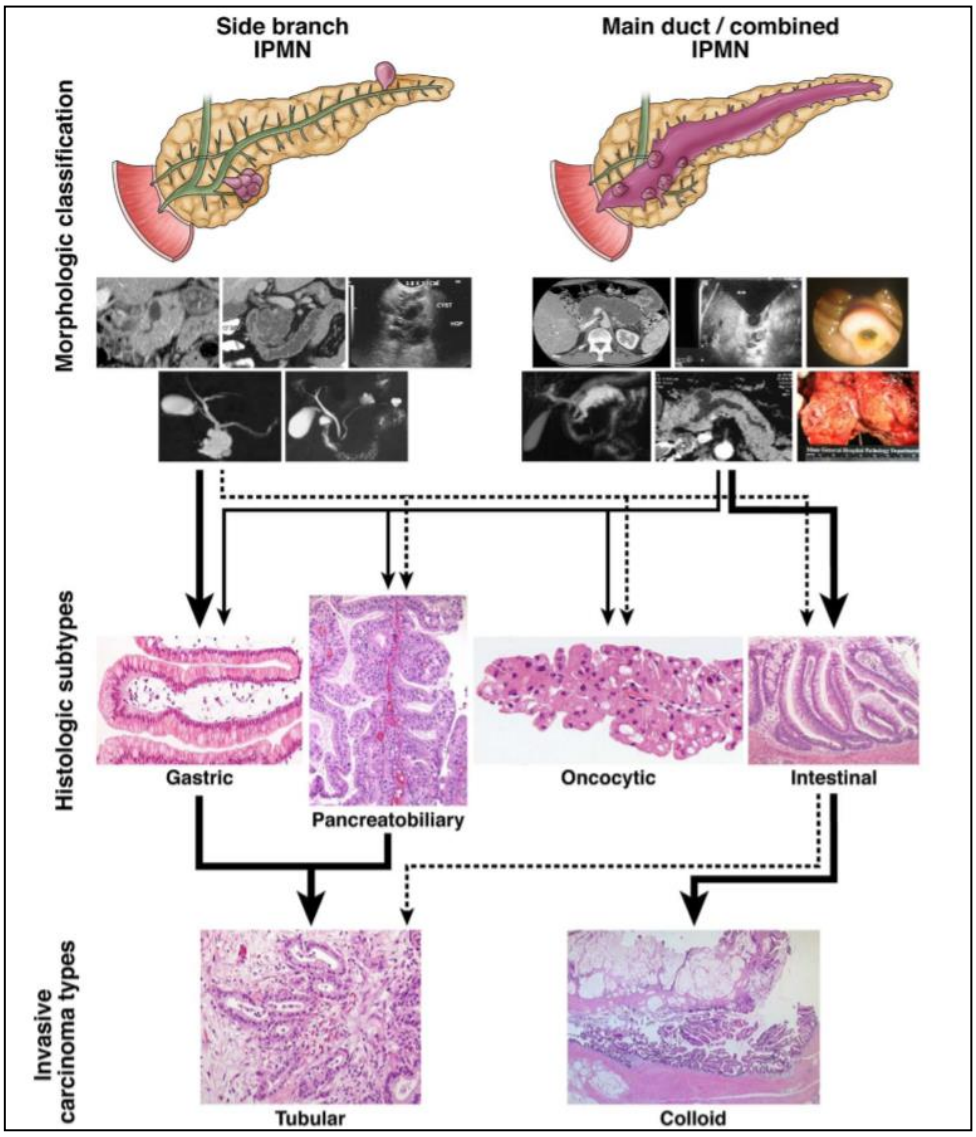


Figure 6. Morphologic and histologic subtypes of IPMNs, as well as the types of invasive cancer. The heavy arrows indicate the most prevalent associations, whereas the thin and dotted arrows indicate less common and rare associations, respectively. By far, BD-IPMNs are the most common, and are mostly of a gastric phenotype. Although the majority do not progress to invasive cancer, when they do, the carcinomas are of the tubular type. By contrast, main and combined duct IPMNs (MT-IPMNs) are mostly of the intestinal type, and most do progress into invasive cancer, which is of the colloid type.

Reproduced with permission from Fernandez-Del Castillo et al. (2010) Intraductal Papillary Mucinous Neoplasms of the Pancreas. Gastroenterology. doi:10.1053/j.gastro.2010.07.025.[103]

Many studies have evaluated PCF as a possible source of markers of HGD or malignancy. Analysis of mucin expression in PCF shows that overexpression of mucine (MUC) 1 is associated with invasive carcinoma and is expressed in pancreatobiliary and oncocytic subtypes. MUC4 is also associated with the grade of dysplasia. Contrary to MUC1, MUC2 expression is not expressed in invasive carcinoma. MUC5A is expressed in intestinal and gastric IPMN subtypes, usually with LGD.[106]

Genomics in PCF started with the PANDA study[1], in which the authors concluded that *KRAS* and *GNAS* mutations were useful for IPMN diagnosis, but were unable to predict the grade of dysplasia.[107,108,109,110] Singhi *et al.*, using preoperative NGS in PCF, confirmed that

KRAS/*GNAS* mutations are highly sensitive for IPMNs and specific for mucinous cyst, and in addition, the combination of *TP53*/*PIK3CA*/*PTEN* mutations are useful markers for advanced neoplasia.[111]

More recently, composite clinical and molecular models have shown promising results on the selection of cysts for surgical resection.[29] Additionally, epigenetic changes have been evaluated, with a panel of methylated markers able to predict the grade of dysplasia of pancreatic cysts.[112] Candidate miRNAs to identify high-grade IPMNs and exclude non-mucinous cysts have also been evaluated, but validation in a prospective setting is still required to ultimately confirm their clinical utility.[113,114] The monoclonal antibody Das-1, which reacts to normal intestinal epithelium, was able to differentiate low- from high-risk IPMNs.[115] The neutrophil-to-lymphocyte ratio (NLR) is a simple, non-invasive blood test, available in clinics, that predicts HGD and invasive carcinoma, presumably reflecting the systemic immune response elicited by cancer, among other potential physiological mechanisms.[102] Further genomics studies in plasma, evaluating the tumor-derived fraction of circulating cell-free DNA (cfDNA), may allow non-invasive diagnosis and monitoring of progression in IPMNs.[116]

These studies have improved our understanding of the natural history of premalignant cysts, with multiple clinical and biological predictors of HGD and malignancy, adding to currently established PCF cytology and imaging features, possibly improving selection for surgery.[10]

Nevertheless, the level of evidence for most published studies is limited, due to the predominance of surgical cohorts, limited sample size, and retrospective design, making high-risk lesions dominant in the literature. These lesions do not represent the daily clinical practice, in which most PCLs are small, benign or low-risk, and require only long-term surveillance.

In clinical practice we need to identify better biomarkers for early detection of rare high-risk lesions and to discharge from surveillance programs those PCLs that have a benign behavior and do not progress. With this strategy, a sustainable increase in early diagnosis of high-risk lesions can be expected, predictably lowering the incidence of advanced IPMN-DCs and improving the unfavorable prognosis of pancreatic cancer.

2.5. Current Guidelines for Management of Pancreatic Cysts

There is probably no other health disorder so prevalent and potentially severe, for which evidence is so low, due to the paucity of randomized trials performed. An attempt to summarize the best available evidence for the clinical management of PCLs has been made by experts in the field with the preparation of several recent guidelines.[81,79,97,98]

Table 4. An overview of the four most recent guidelines on the diagnosis and management of PCLs.
Reproduced with permission from Levink IJM et al. (2018) Management of Intraductal Papillary Mucinous Neoplasms: Controversies in Guidelines and Future Perspectives. Curr Treat Options Gastro. Doi: 10.1007/s11938-018-0190-2.[68]

	Revised EU guideline (2018)	Revised Fukuoka guideline (2017)	ACG guideline (2018)	AGA guideline (2015)
Diagnostic work-up	MRI: 1st choice CT: 2nd choice* EUS: supplementary FNA: in case of mural nodules, septations or indefinite imaging Serum 19-9	MRI: 1st choice CT: 2nd choice* EUS: for worrisome features FNA: in case of indefinite imaging; discouraged in case of high-risk/ worrisome features Serum 19-9	MRI: 1st choice EUS/CT: alternative FNA: in case of indefinite imaging, high risk characteristics, cysts > 2 cm (differentiation mucinous and non-mucinous) Serum 19-9	MRI: 1st choice EUS: high-risk features FNA: in case of ≥ 2 high-risk features or significant change of high-risk feature
MD-/MT-IPMN: indications for surgery	Surgically fit patients	Surgically fit and ≥ 1 high-risk stigmata (see below)	Reference to multidisciplinary group in case of main-duct involvement	Not mentioned
BD-IPMN: high-risk features/indications surgery	Absolute indications: Solid mass Enhancing mural nodule ≥ 5 mm MPD ≥ 10 mm HGD/carcinoma in cytology Jaundice Relative indications: Cyst growth ≥ 5 mm/year Cyst size ≥ 4 cm Enhancing mural nodule < 5 mm MPD 5–9.9 mm Serum CA 19-9 ≥ 37 U/ml New-onset DM Acute pancreatitis	High-risk stigmata: Enhancing mural nodule > 5 mm MPD > 10 mm Jaundice Worrisome features: Growth ≥ 5 mm/2 years Cyst size ≥ 3 cm Enhancing mural nodule < 5 mm Enhancing thickened cyst wall MPD 5–9 mm PD calibre change Elevated serum CA 19-9 Pancreatitis	High-risk characteristics: Mural nodule/solid component MPD > 5 mm PD calibre change + atrophy Cyst size ≥ 3 mm Cyst growth > 3 mm/year HGD/carcinoma in cytology Jaundice Acute pancreatitis Elevated serum CA 19-9 New-onset DM	High-risk features: Solid component Dilated MPD Cyst size ≥ 3 cm
Duration surveillance	As long as fit for surgery	As long as fit for surgery	As long as fit for surgery Individualized approach for age 76–85 years	Discontinue after 5 years if no significant change has occurred
Surveillance intervals	6 months (1st year), then yearly	< 1 cm: 6 months, then 2 yearly 1–2 cm: 6 months (1st year), yearly (2 years), then 2 yearly 2–3 cm: 3–6 months (1st year), then yearly > 3 cm: 3–6 months	< 1 cm: 2 years 1–2 cm: 1 year 2–3 cm, clear IPMN/MCN: 6–23 months. Shorter interval for new-onset DM or cyst growth > 3 mm/year	At years 1, 3 and 5
Indication for surgery	≥ 1 Absolute indication ≥ 1 Relative indication without significant co-morbidities ≥ 2 Relative indications for patients with significant co-morbidities	≥ 1 High risk stigmata ≥ 1 Worrisome feature and ≥ 1 of the following: Definite mural nodule, MPD involvement Suspect cytology Consider: cyst > 2 cm in young and fit patient	Decided by multidisciplinary team. Referral in case of jaundice or ≥ 1 of the following: MPD > 5 mm, Cyst size ≥ 3mm Calibre change MPD MPD involvement HGD/PDAC cytology Mural nodule	Solid component and dilated MPD and/or concerning features on EUS-FNA

The Sendai consensus, published in 2006, established the earliest guidelines for management of IPMNs and MCNs.[48] These were revised in 2012[7] and later in Fukuoka in 2017[79], and have been widely adopted for surgical decision-making. Progressively, the guidelines have become more surgery-restrictive because surgical specimens were predominantly low-grade.

In the 2017 Fukuoka guidelines the absolute indications for surgery were restricted to "high-risk stigmata" (jaundice, enhanced mural nodule ≥5 mm, or main duct dilatation ≥10 mm), with EUS-FNA for cytology recommended for "worrisome features" (cyst ≥3 cm, thickened/enhancing cystic walls, main pancreatic duct 5-9 mm, non-enhancing mural

nodule, abrupt change in caliber of pancreatic duct with distal pancreatic atrophy, lymphadenopathy). However, these recommendations are based on the premise that we can accurately classify PCLs with demographics, clinics, morphology, and PCF analysis. However, in clinical practice, the preoperative diagnosis often differs from that of the surgical specimen. Similarly, the absolute indications for surgery in the Revised European Guidelines include only a positive cytology for malignant/high grade dysplasia, solid mass, jaundice, enhanced mural nodule ≥5 mm, or main duct dilatation ≥10 mm.[98]

When referring for surgery asymptomatic PCLs, the main differences between earlier and existing guidelines concern the thresholds of pancreatic cyst and Wirsung diameters. When referring to BD-IPMNs, the 2018 Revised European (EU) guidelines[98] do not consider the size of the peripheral cyst among the factors representing an absolute indication for surgery, although a size of 40 mm represents a relative indication to be combined with other cyst and patient features. The Revised Fukuoka guidelines[79] consider a diameter >30 mm as a "worrisome feature" requiring further investigation or eventually a surgical indication in young individuals. The American Gastroenterological Association (AGA) guidelines[81] consider three possible high-risk features (i.e. cyst size >30 mm, dilated Wirsung duct, or the presence of a solid component) with the presence of at least two being required for further evaluation by EUS. In any case, the size of a BD-IPMN *per se* should not be considered as an absolute indication for surgery.

With regard to the diameter of the Wirsung duct, the cut-off of 10 mm is an indication for surgery according to the EU[98] and Revised Fukuoka guidelines[79], while the AGA generically mention "Wirsung duct dilation". The Revised EU guidelines also include a diameter above 5 mm among "relative indications" for surgery according to studies reporting an increased rate of malignancies with a Wirsung duct diameter >5 mm.[98]

In fact, the larger is the diameter of the Wirsung duct, the greater is the risk of malignancy, but the rate of IPMNs with a dilation >10 mm is rather small. Therefore, the fraction of patients is limited, making this risk feature nonsignificant in clinical practice. Furthermore, data supporting these different policies are obtained in retrospective surgical series with all inherent biases. Moreover, it is unclear how this delicate "millimeters' cutoff" should be measured, given the reported low agreement between MRI and EUS in reporting the size of both BD-IPMNs and Wirsung duct.[117]

The AGA guidelines[81], are especially controversial about who should undergo surgery, as resection is recommended only for patients with two of three concerning features (e.g. size >30 mm, a mural nodule, or MPD dilation) *and* with evidence of malignancy after EUS. Still, the authors recognize that the sensitivity of EUS-guided cytological analysis is only 60%, meaning that 40% of patients with cancer would be missed if these criteria were applied. Low sensitivity of AGA guidelines was confirmed in both asymptomatic[118] and symptomatic PCLs.[30] Another critical aspect is the recommendation to stop surveillance after IPMN resection and in non-resected IPMNs, after 5 years in the event that no cystic changes occur, which runs

against the evidence on the risk of recurrence of IPMN after resection and ignores increased incidence of PDAC in patients with mucinous cysts.[104,95,119]

With the recent guidelines of the American College of Gastroenterology (ACG)[97], there is an attempt of tailoring the decision to the patient, with recommendation to withhold evaluation or surveillance of incidental pancreatic cysts in patients medically unfit for surgery or older, regardless of cyst size, and to stop surveillance of lesions with very low risk of malignant transformation, such as SCAs.

Among the important limitations of current guidelines are the low quality of evidence presented, being "cyst centered", relying on surgical cohorts for diagnosis with selection bias, and possible overfitting bias, since in clinics most PCLs require only surveillance.

In summary, guidelines currently available are useful for identifying patients at risk of developing IPMN-DCs with a high level of sensitivity. Due to reduced specificity, however, non-malignant PCLs are often resected, exposing patients to unnecessary morbidity and mortality related to pancreatic resection. The results of observational studies on cohorts of patients under surveillance have highlighted that most PCLs can be safely observed due to low risk of progression. It is not yet possible to know whether a surveillance protocol is associated with a reduction in pancreatic cancer related mortality, but continuation of follow-up must be recommended due to the lifelong risk of IPMN-DCs.

Additional factors besides cyst morphology, including clinical data and biomarkers, will be required to improve our understanding of cyst behavior.[29] Patients with advanced age or comorbidities have an increased mortality from fragility and pancreatic resection, rather than from cyst malignancy. The shaping role of Charlson comorbidity index for decision was explored by some authors[120,121], confirming that other aspects beyond cystic features affect survival.

3. New Biomarkers for Diagnosis of Pancreatic Cystic Lesions

Recent developments in areas of research such as genomics and proteomics offer new approaches to early cancer detection. A framework for advancing biomarkers from the laboratory to the bedside has been previously proposed.[36] This framework is valid for development of biomarkers in PCLs.

3.1. Biomarkers from Bench to Bedside

The process of biomarker development in cancer is ideally categorized in five phases, well characterized by Thiruvengadam N *et al.*[36], that are necessary for a biomarker to pass through, until it produces a useful clinical tool, as illustrated in Figure 7.

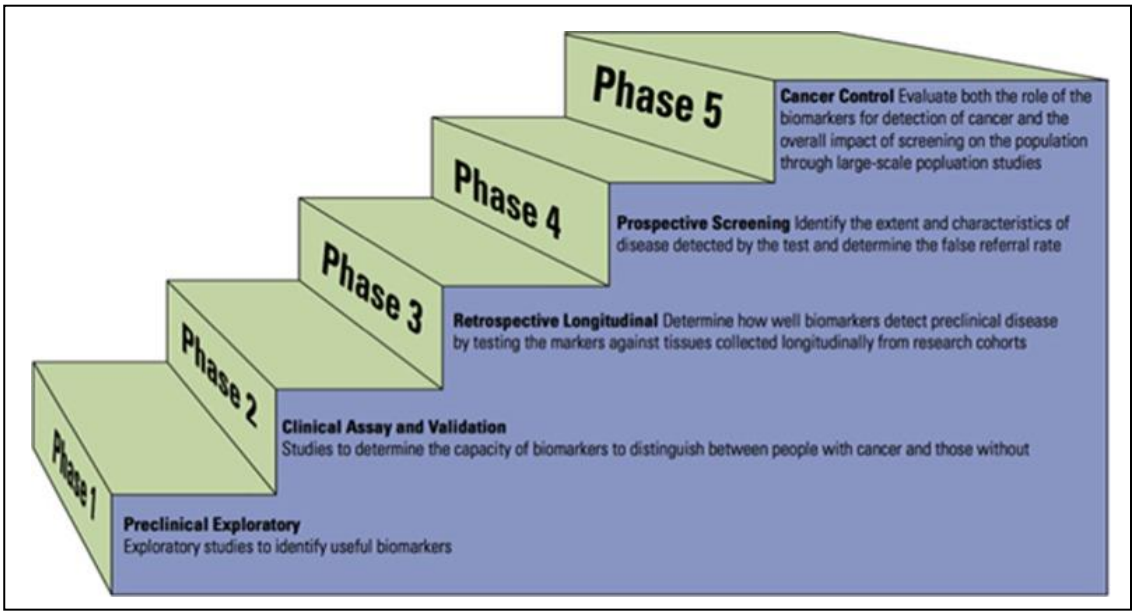


Figure 7. Graphic representation of the five proposed phases for diagnostic test discovery and validation.
As defined by Pepe *et al.*[122]: in phase 1 (Preclinical Exploratory), promising directions are identified. In phase 2 (Clinical Assay and Validation) the clinical assay detects an established disease; in phase 3 (Retrospective Longitudinal) the biomarker detects disease early before it becomes clinical and a "screen positive" rule is defined; in phase 4 (Prospective Screening) the extent and characteristics of the disease detected by the test and the false referral rate are identified; and in phase 5 (Cancer Control) the impact of screening on reducing the burden of disease on the population is quantified.
Reproduced with permission from Thiruvengadam N et al. (2015) Systematic Review of Pancreatic Cyst Fluid Biomarkers: The Path Forward. Clinical and Translational Gastroenterology. doi:10.1038/ctg.2015.17.[36]

Most published studies of biomarkers in PCF are classified in phases 1 and 2 with the most important phase 3 studies being based on repositories from centers that have already been banking PCF. Those samples can be collected during surgery, but also by EUS-FNA, in lesions that immediately or eventually in the future will undergo surgery. The goal is to assess biomarkers' capacity to predict disease outcome, which is assessed retrospectively.

With the proper design and application, retrospective longitudinal studies can allow for rapid clinical uptake of novel biomarkers. In fact, successful phase 3 studies allow the implementation of biomarker to proceed into clinical practice. These studies should include minimally acceptable true- and false-positive rates for testing defined by community consensus.

With our current knowledge of the indolent nature of most of premalignant cysts, phase 4 prospective studies, while important, require a very large sample with longitudinal follow-up that may not be practical or feasible in order to define as threshold prior to clinical use.

Finally, phase 5 studies address whether or not screening reduces the burden of cancer on the population.

3.2. Composite Markers

Cancer is a heterogeneous disease, and it is unlikely that a single biomarker might detect a specific malignancy with high specificity and sensitivity. High sensitivity is fundamental for cancer biomarkers, with false-negative results leading to delayed medical care and poorer prognosis. Maintaining high specificity (low false-positive rates) is also a high priority for cancer detection, particularly in PDAC. Even a small false-positive rate translates into many patients subjected to costly and invasive diagnostic procedures, psychological stress, and even unnecessary surgeries related to concern for the dismal prognosis of PDAC. Biomarkers need to be extremely sensitive and specific and the simultaneous use of several different biomarkers may be necessary for a sensitive and specific diagnosis.

To aid in the diagnosis of PCLs, composite markers appear to be especially useful. These combine multiple individual parameters into a single marker. Composite marker selection usually requires the use of algorithms.

In summary, the imaging identification of two macroscopic precursors to PDAC, i.e. IPMNs and MCNs offers the potential for early detection. In clinical practice this approach is challenging. First, there are many different cyst types, some without risk of malignant transformation, like SCAs or pseudocysts. Second, most IPMNs and MCNs will not progress into invasive cancer. Thus, in clinical practice, the key clinical questions are: i) how to differentiate IPMNs and MCNs from benign PCLs that require no follow-up; ii) how to identify high-risk IPMNs and MCNs that require surgery. The tools currently available, CEA and cytology in PCF, are imperfect in answering these questions and more accurate tests are needed.

One avenue that has been explored is whether molecular markers, i.e. analysis of DNA, RNA, proteins, or metabolomic changes within the cyst, could aid in the diagnosis and management of PCLs. In the last 10 years, genetic analyses have examined the somatic mutations that occur in each type of PCLs with all types of pancreatic cystic neoplasms analyzed by whole exome sequencing, providing great insights into the genetic alterations underlying each PCL.[108] These studies have revealed that different histologic cyst subtypes possess unique combinations of driver genes that genetically mirror cyst morphology (Table 5), highlighting the somatic mutations as potential biomarkers useful in preoperative diagnosis.

Table 5. Genes mutated in the most common precursors of PDAC and pancreatic cystic neoplasms.
Reproduced with permission from Lennon AM et al. (2014) The Early Detection of Pancreatic Cancer: What Will It Take to Diagnose and Treat Curable Pancreatic Neoplasia? Cancer Research. doi: 10.1158/0008-5472.CAN-14-0734[11]

Gene	IPMN-LG	IPMN-HG	MCN-LG	MCN-HG	PanIN-1 and -2	PanIN-3	SCA	SPN
KRAS	X	X	X	X	X	X		
P16/CDKN2A	X	X	X	X	X	X		
TP53		X		X		X		
SMAD4		X		X		X		
RNF43	X	X	X	X				
GNAS	X	X						
CTNNB1								X
VHL							X	

Abbreviations: HG, high grade; LG, low grade.

3.3. New Biomarkers in Clinical Practice

The promise of molecular markers for the diagnosis and management of PCLs has several issues to address. The first is how to incorporate multiple different markers together, and how to combine them with clinical features. The second is study quality, with much of the current data coming from small, retrospective, single-center studies with non-reproducible findings.

In early studies, the performance of the presumptive future markers should be compared with the gold standard, i.e. surgical pathology data, with the promising markers then evaluated in prospective, multicenter studies. To be incorporated into clinical practice, a marker panel needs to show analytical reproducibility, clinical validation, and clinical utility, and must improve the quality of life and also to be cost-effective.

Chapter II.

Rationale and Research Questions

The growing frequency of asymptomatic PCLs[2,3], ranging from benign to pre-malignant and malignant lesions, represents an opportunity for early diagnosis of PDAC. It involves the precise identification of high-risk and early malignant cystic neoplasms in order to improve patient selection for surgical resection, which should be performed early in high-grade lesions and avoided in low-grade ones.

No accurate methods are currently available for a reliable triage of these patients, based on morphology or PCF analysis only.[123]

For the purpose of this dissertation we reviewed the utility of distinct biomarkers on the differential diagnosis of pancreatic cysts and studied specific biomarkers in PCF obtained by EUS-FNA to identify significant PCLs, namely mucinous (pre-malignant) and high-risk/malignant cysts. We studied a cohort of patients selected from the PCF EUS-FNA database of the *Instituto Português de Oncologia de Lisboa de Francisco Gentil, EPE* (IPOLFG-EPE), started in 2008, with a longitudinal follow-up of 9 years.

The following questions were addressed:

a. Is the differential diagnosis of pancreatic cysts improved by molecular analysis, including *KRAS* and *GNAS* mutations?
b. Is the identification of pancreatic mucinous cysts improved with the assessment of glucose level in PCF using a glucometer comparing to CEA?
c. Is a second EUS-FNA in PCLs under surveillance an asset for the differential diagnosis of cysts?
d. Is EUS-FNA with PCF analysis helpful to evaluate small (<3 cm) PCLs?
e. Is the evaluation of Chromogranin A and NSE in PCF useful for the diagnosis of cystic pancreatic NETs?
f. Is methylation analysis of complex *locus GNAS* helpful for diagnosis of high-risk/malignant PCLs?

Chapter III.

Material and Methods

1. Study Design

Our study strategy included a review of the literature, with two meta-analyses, in order to guide and support the original research performed on the specific biomarkers.

Our original research was based on longitudinal retrospective cohort studies of patients with PCLs referred for EUS-FNA over time, with clinical data, imaging, and standard PCF analysis reviewed in order to evaluate their diagnostic value in our cohort of patients, composed mainly of low-risk PCLs with extended imaging surveillance. Additionally, using PCF samples collected and stored at inclusion in the PCF database, we assessed the accuracy of specific biomarkers in this target population

Our comprehensive approach of biomarkers in PCF included genomics (*KRAS and GNAS* mutations), epigenomics (methylation analysis of *GNAS* complex *locus*), proteomics (CEA), metabolomics (glucose, CroA, NSE), and repeating cytology.

The accuracy of some of the most promising biomarkers was evaluated in a surveillance cohort, representative of current clinical practice. With this validation approach in this population, we sought to avoid overfitting, in which a classification model performs well in a training sample (prior studies performed in surgical cohorts) but performs poorly when applied to new data (predominantly clinical cohorts).

2. Study Plan and Methodology

We addressed PCLs from different perspectives, divided according to specific aims, disclosing a comprehensive approach.

2.1. Molecular Analysis for Assessment of Pancreatic Cysts

Rationale: The DNA mutational profile of PCF may reliably classify cysts into mucinous and non-mucinous subtypes.

Aim 1: To review the literature and evaluate the recognized additional value of *KRAS* mutation for diagnosis of pancreatic mucinous cysts.

Aim 2: To review the literature and to compare the performance of *KRAS* mutational analysis with MFB of the cyst wall for diagnosis of PCLs.

2.2. Biomarkers for Diagnosis of Mucinous Pancreatic Cysts

Rationale: The combination of *KRAS* and *GNAS* mutations was suggested as a very sensitive approach for early diagnosis of mucinous cysts, known precursors of PDAC, with the drawbacks of cost and complexity.

Glucose level assessed by a glucometer was suggested to be a sensitive biomarker to identify mucinous cyst as it is simple, cheap, and requires a minimal amount of PCF.

Aim 1: To evaluate additional value of *KRAS and GNAS* mutation analysis in the diagnosis of PCLs in a cohort of patients with previous standard analysis of PCF.

Aim 2: To compare the performance of glucose level using a glucometer with standard CEA analysis for diagnosis of pancreatic mucinous cysts.

2.3. Biomarkers for Diagnosis of High-risk and Malignant Pancreatic Cysts

Rationale: High-risk and malignant pancreatic cysts include several entities with difficult differential diagnosis.

Cyst size above 3 or 4 cm has been considered a high-risk feature, but the rationale for this limit is debatable. Also, the role of a second EUS-FNA on a PCL without worrisome features is rarely discussed.

NETs may be cystic, with non-diagnostic PCF analysis except cytology for pre-operative diagnosis with possible false-negative results.

Methylation has a recognized role in cancer. Nevertheless, while *GNAS* mutation, an early event in IPMNs that is detectable in PCF, methylation changes at *GNAS locus* have not been previously studied in PCLs.

Aim 1: To assess the value of a second EUS-FNA for the diagnosis of malignancy in PCLs.

Aim 2: To assess the value of EUS-FNA in the evaluation of PCLs smaller than 3 cm.

Aim 3: To assess the value of Chromogranin A and NSE levels in PCF for the diagnosis of cystic NETs.

Aim 4: To assess the role of methylation of *GNAS complex locus* in malignant PCLs.

3. Patients

For original work we selected patients from the registry of PCF EUS-FNA database of the IPOLFG started in 2008, which is used for diagnosis and clinical management of patients with pancreatic cysts. All patients gave informed consent for EUS-FNA and PCF analysis and storage. The studies of different biomarkers were approved by the Ethics Committee and Institutional Scientific Board of IPOLFG, EPE (UIC/1143, UIC/1224 and UIC/1225).

From 266 patients undergoing EUS-FNA for pancreatic cyst evaluation between 2008 and 2014, 102 frozen PCF samples were obtained, stored at -80˚C, and were available for our biomarker research. Clinical data, including cyst characteristics and treatment decision, had been prospectively collected and registered, with all prospective data evaluated in 2016. The accuracy of biomarkers was evaluated by cyst type and by groups of cysts, according to surgical pathology (surgical cohort) or EUS-FNA cytology diagnosis and prolonged clinical follow-up (clinical cohort).

4. EUS-FNA for PCF Collection and Storage

In all patients undergoing EUS-FNA for evaluation of a pancreatic cyst, the PCF obtained is immediately separated into two samples. Sample A (0.5 mL) is centrifuged for

cytospin preparation for cytological analysis, and the supernatant fluid is sent for CEA and amylase evaluation; sample B (with the remaining volume of PCF) is immediately put on ice and stored at -80˚C in 0.25 mL aliquots, no more than 30 minutes after collection.

Chapter IV.

Results

1. Molecular Analysis for Assessment of Pancreatic Cysts

1.1. *KRAS* in Cyst Fluid Obtained by EUS-FNA in Pancreatic Cystic Lesions: A Systematic Review and Meta-analysis. Published in Pancreas.

Abstract

To evaluate the diagnostic accuracy of *KRAS* mutation in pancreatic cystic fluid and compare it with Carcinoembryonic Antigen (CEA) and cytology, we identified studies with cyst fluid obtained by Endoscopic Ultrasound (EUS) prior to surgery. We classified cysts as malignant, pre-malignant, and benign. A random effects model was used for quantitative meta-analysis. Pooled sensitivities, specificities, and summary receiver operating characteristic (SROC) curve analysis were conducted. We analyzed sixteen studies, with 3429 patients, including 731 referred for surgery. CEA was the best test for clinically significant cysts (pre-malignant and malignant) with sensitivity = 0.58 (95% CI, 0.53-0.65), specificity = 0.9 (95% CI, 0.76-0.97), and area under the curve (AUC) = 0.69. Cytology performed better in malignant cysts, with sensitivity = 0.37 (95% CI, 0.27-0.48), specificity = 0.96 (95% CI, 0.93-0.98), and AUC = 0.78. Isolated, *KRAS* mutation failed the diagnosis of malignant and significant cysts, with sensitivities = 0.43 (95%CI, 0.34-0.43) and 0.46 (95% CI, 0.42-0.51), specificities = 0.62 (95% CI, 0.56-0.68) and 0.97 (95% CI, 0.92-0.99), and AUC = 0.56 and 0.53, respectively. As CEA and cytology are more accurate than *KRAS*, it should be a complementary test. Additional studies are lacking to recommend *KRAS* as a single diagnostic test.

Key words

Pancreatic cyst, KRAS, cea, cytology, eus, eus-fna

Text

1. INTRODUCTION

Pancreatic cystic neoplasms (PCNs) are increasingly found in clinical practice, due to an ageing population and the routine use of high-quality abdominal imaging.[1] The importance of PCNs is related to malignant potential, high frequency, and significant morbidity and mortality of surgical treatment. Therefore, there is an urgent need to find non-invasive and reliable markers of malignant and high-risk pre-malignant PCNs.

In clinical practice, after clinical and imagiological findings of a potentially significant lesion, including mucinous pre-malignant or malignant cysts, endoscopic ultrasound with fine-needle aspiration (EUS-FNA) for cystic fluid analysis for CEA and cytology became standard in decision-making. CEA is the most accurate for diagnosing mucinous cysts, which are pre-malignant lesions, whereas cytology is highly specific for malignancy diagnosis.[2] Treatment options, including surgery, follow-up, or no additional evaluation, rely on imaging and PCF analysis, but a significant part remains indeterminate, with about one third of pre-operative diagnosis being incorrect.[3,4]

In this clinical context, pancreatic cyst fluid analysis for molecular markers has shown that *KRAS* mutations may be specific for mucinous cysts[5,6,7] and that simultaneous *KRAS*/*GNAS* mutations are specific of intraductal papillary mucinous neoplasms (IPMNs).[8,9] Currently, next-generation sequencing (NGS), a very sensitive technique for detection of genetic mutations, can be considered in indeterminate PCNs or if it modifies patient management.[10] Numerous studies have shown that DNA

molecular analysis of aspirates obtained by EUS-FNA provide a better characterization of PCNs comparing to current methods used in clinics.[11,12,13,14,15,16,17,18,19] However, these studies have generally included a limited number of patients and results are not consistent among studies. Currently, the integration of molecular analysis in routine clinical practice is still a matter of debate.

We therefore performed a systematic review and meta-analysis of all previous studies with *KRAS* mutational analysis performed by NGS in pancreatic cystic fluid obtained pre-operatively by EUS-FNA. All samples with a surgical pathology as reference standard for diagnosis were evaluated. Our aim was to investigate the accuracy of *KRAS* mutational analysis for diagnosis of mucinous and significant (mucinous and malignant) PCNs and compare it to routine standard diagnosis, with CEA and cytology.

2. METHODS

Search strategy and eligibility criteria

The systematic review and meta-analysis reported here were conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines,[20] and the protocol was registered at PROSPERO (CRD42018097268). A comprehensive search of databases, including Medline, Scopus, Web of Science, and Scielo for the past 18 years (January 1st, 2000 to March 31st, 2018) and restricted to human studies was performed. No language restrictions were applied. The following search terms were used: “pancreas”, “cyst”, “molecular”, and “analysis”. Additional search of related articles and hand-search of references of all selected studies was performed, adding additional publications.

Inclusion criteria: Published studies were included in the meta-analysis if they: 1) Analyzed mutational analysis of *KRAS* using highly sensitive techniques, such as NGS; 2) Analyzed a cohort of patients with pancreatic cysts, symptomatic or incidental findings; 3) Cysts were evaluated by EUS-FNA with PCF analysis; and 4) All patients had a definitive diagnosis with a surgically resected specimen.

Exclusion criteria: 1) Studies on molecular markers other than *KRAS* mutation; 2) Studies involving solid pancreatic lesions; 3) Studies performed in PCF not obtained by EUS-FNA; 4) Studies with cytology and clinical information as standard criterion of diagnosis without a surgical pathology specimen as reference standard; and 5) Reviews, case reports, letters to editor, exploratory studies, and papers published only in abstract form.

Two reviewers (SF and AL) independently judged study eligibility and disagreements were resolved by consensus.

Histological criteria and tests under investigation

Based on WHO tumor classification, PCNs diagnosis were reviewed and classified into one of three groups: 1) malignant cysts (adenocarcinoma or high-grade dysplasia in IPMNs and MCNs, secondary cystic adenocarcinomas, and cystic pancreatic neuroendocrine tumors - PNETs); 2) pre-malignant mucinous cysts (IPMNs and MCNs with low or intermediate-grade dysplasia); 3) benign cysts (serous cystadenomas, pseudocysts, and other benign cysts).

The index test was molecular analysis with *KRAS* mutation, because it is the most frequent mutation. The comparators were: 1) CEA (cut-off value above 192 ng/mL) for diagnosis of mucinous cystic lesions; and 2) Cytology that was considered positive if samples were read as atypical, suspicious, positive, or malignant. Cytology was considered negative if samples were read as indeterminate, acellular, or negative for malignancy. It should be noted that a diagnosis of atypia in a cytological evaluation does not warrant a malignancy diagnosis requiring surgery.

Outcomes

The primary outcome was to assess the diagnostic accuracy of *KRAS* mutation in PCF for diagnosis of malignant and significant PCNs. The secondary outcome was to compare the accuracy of *KRAS* mutation with current standard of diagnosis, with PCF analysis for CEA and cytology, in malignant and significant PCNs.

Data Extraction and quality assessment

Selected articles' data were extracted independently by two reviewers (SF and AL), who were blinded to publication details, onto a predefined worksheet. Disagreements were discussed and reviewed by a third reviewer (LP).

Data extraction included the name of first author, publication year, study design (prospective, cross-sectional, retrospective), sample size (all patients included in the study), number of patients referred for surgery (surgical cohort), number of malignant lesions, distribution of cyst types (malignant, pre-malignant, benign), number of patients with a CEA >192ng/mL, a positive cytology, and *KRAS* mutation detection.

Methodological quality of primary studies included was assessed by two authors (SF and AL) using the modified QUADAS-2 tool[21], which evaluates the quality of articles for systematic reviews of diagnostic accuracy studies in four domains, including patient selection, index test, reference standard, and flow and timing, for risk of bias and applicability concerns.

Statistical Analysis and Data Synthesis:

Our reference standard was surgical specimen that classified PCNs into three groups: malignant, pre-malignant, and benign cysts. This resulted in a two-by-three table: positive or negative test result in each of the three groups, for each of the three tests, *KRAS* (index test), cytology, and CEA (comparator tests).

To calculate test accuracy and to reflect the categories that are used in clinical practice and guide management, we constructed two-by-two tables, to evaluate the ability of the index test and comparator tests to discriminate malignant from non-malignant (all cysts except those proven to be malignant) and significant (proven malignant and pre-malignant cysts) from non-significant cysts (proven benign cysts).

The data of the two-by-two tables were used to calculate sensitivity and specificity for each study. We present individual study results graphically by plotting the estimates of sensitivity and specificity (and their 95% confidence intervals (CI)) in both forest plots and on the summary receiver operating characteristic (sROC) curve plots. The area under the curve (AUC) is equal to the probability that if a pair of relevant and non-relevant cysts is selected at random, the relevant cyst will have a higher or positive test result than the non-relevant cyst. Pooled estimates of the sensitivity and specificity were obtained by DerSimonian-Laird method (random effect model) to incorporate variation among studies, when data are heterogeneous.

Heterogeneity was investigated in the first instance through visual examination of forest plots of sensitivities and specificities and through visual examination of the ROC plot of the raw data. Last, we used the chi-square test to evaluate if the differences across the studies were greater than expected by chance alone. A low p-value suggested presence of heterogeneity. In addition we used the statistic I^2 of Higgins that allowed us to quantify the amount of heterogeneity.[22,23] The scale of I^2 has a range of 0 to 100% and values of 25%, 50%, and 75% are considered low, moderate, and high heterogeneity.

Publication Bias

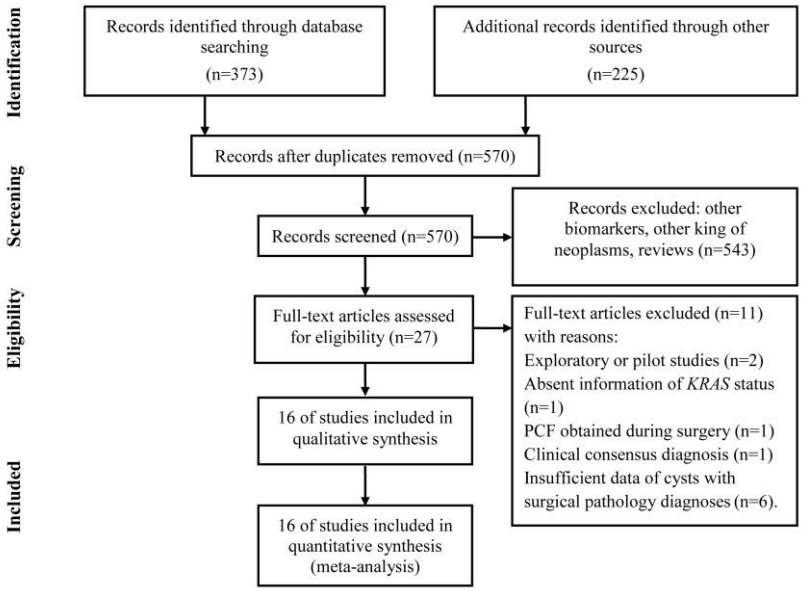
To analyze the publication bias in meta-analyses of sensitivity and specificity we used Deeks' test. This test, developed for diagnostic test accuracy (DTA), is the least biased and is recommended in the Cochrane DTA Handbook.[24,25]

We used Meta-DiSc (version 1.4 – Meta-Analysis of Diagnostic and screening tests)[26] for assessment of diagnostic yield of the studies and SPSS Statistics (version 23, IBM corporation) for Deeks' test.

3. RESULTS

Search results and characteristics of the studies included

Our search found 496 study titles and abstracts. Figure 1 describes the selection process of the articles included in this study.



After abstract screening and full-text review, 16 studies met the inclusion criteria and were considered suitable for qualitative and quantitative analysis. The design was retrospective in 13 and prospective in three articles, with three studies published from years 2005 to 2009, eight from years 2010 to 2014 and five from years 2015 to 2018.

These 16 studies included a total of 3429 patients, of which 731 (21%) underwent surgical resection and had a surgical pathology specimen available as reference standard and were included for analysis. Patients of studies in which data were available for the overall series (aggregating results of surgical and clinical surveillance cohorts) but were not discretely available for the surgical cohort, and were excluded from analysis.

The characteristics of the studies, surgical pathology diagnoses and cystic fluid analysis details are in Table 1.

Table 1. Characteristics of Studies and Test Results Included in the Analysis

1st Author, year	Prospective/Retrospective (Study period)	Diagnosis	Sample size (all patients in the study)	Surgical cohort	Malignant cysts (surgical pathology)	Mucinous pre-malignant Cysts (surgical pathology)	Benign Cysts (surgical pathology)	CEA>192 ng/mL	Positive Cytology	*KRAS* mutation	CEA≥192 in Malignant/ Mucinous/Benign	Cytology+ in Malignant/ Mucinous/Benign	*KRAS* mutation in Malignant/ Mucinous/Benign
Schoedel et al, 2006[12]	Retrospective (NA)	Pathology	16	16	4	12	0	NA	2	4	NA	2/0/0	2/2/0
Sreenarasimhaiah et al, 2009[13]	Retrospective (Jul 2006-Nov 2007)	Pathology	60, 20 study cohort	6	6	0	0	4	0	2	4/0/0	0/0/0	2/0/0
Sawhney et al, 2009[15]	Retrospective (2006-2007)	Pathology or cytology	111, 100 study cohort	19	5	12	2	14	1	2	A/B/0 A+B=14	1/0/0	1/1/0
Mertz et al, 2011[19]	Retrospective (May 2007-March 2008)	Pathology or cytology	60 study cohort	10	0	7	3	NA	5	4	NA	0/4/1	0/3/1
Toll et al, 2010[27]	Retrospective (2007-2010)	Pathology or cytology	63	2	1	0	1	NA	1	1	NA	1/0/0	1/0/0
Panarelli et al, 2012[28]	Prospective (2005-2010)	Pathology	18*	4	1	3	0	1	1	1	0/1/0	1/0/0	0/1/0
Rockacy et al, 2013[29]	Retrospective (NA)	Pathology or cytology	134	51	10	18	16	NA	10	NA	NA	10/0/0	10/NA/NA
Nikiforova et al, 2013[30]	Retrospective (Nov2006 - Oct 2012)	Pathology or cytology	603	142	31	85	26	NA	NA	53	NA	NA	7/46/0
Al-Haddad et al, 2014[31]	Prospective (2008-2012)	Pathology or cytology	286	48	6+4	32	6	24†	7	16†	A/B/0 A+B=24	NA/NA/NA	C/D/NA C+D=16
Singhi et al, 2014[9]	Retrospective (2006-2013)	Pathology or cytology	91	83	19	57	7	36	NA	36	36	NA	8/35/0‡
Kung et al, 2014[32]	Retrospective (2010-2013)	Pathology or cytology	72	6	5	0	1	4	2	2	4/0/NA	2/0/0	2/0/NA
Winner et al, 2015[33]	Retrospective (2006-2012)	Pathology	200	40	10+3	23	4	19	3§	16	A/B/1 A+B=18	2/A/B A+B=1	4/A/B A+B=12
Jones et al, 2015[34]	Prospective (Mar 2013-Feb 2014)	Pathology or cytology	86	10	6	4	0	5	3	8	3/2/0	3/0/0	4/4/0
Singhi et al, 2016[35]	Retrospective (Jan 2014-May2015)	Pathology or clinicopathologic	225	41	13	13	15	NA	6	17	NA	5/0/1	10/7/0
Kadayifci et al, 2016[5]	Retrospective (2006-14)	Pathology or clinicopathologic	943	147	25+12	83	27	72	NA	50	A/B/2 A+B=70	NA	C/D/0 C+D=50
Singhi et al, 2017[7]	Prospective (Jan 14-Jul-17)	Pathology or cytology	595	102	19+9	47	27	NA	7	47	NA	7/0/0	16/31/0

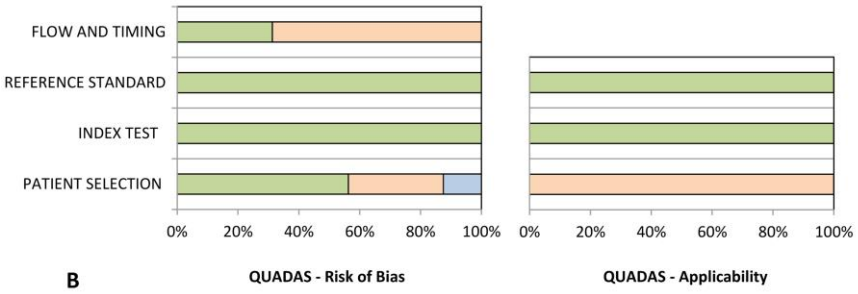
NA-non-available data; *20 cysts in 18 patients; †In 38 pre-malignant mucinous cysts, ‡*GNAS* or *KRAS* mutation, §atypical cytology was grouped as benign in this study.

Quality assessment

Results of methodological quality of the primary studies included are presented in Figure 2, which was sketched with templates available at www.quadas.org.

Study	RISK OF BIAS				APPLICABILITY CONCERNS		
	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD	FLOW AND TIMING	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD
Schoedel[12], 2006	☺	☺	☺	☺	☹	☺	☺
Sreenarasimhaiah[13], 2009	☺	☺	☺	☹	☹	☺	☺
Sawhney[15] , 2009	☺	☺	☺	☹	☹	☺	☺
Mertz[19], 2011	☺	☺	☺	☹	☹	☺	☺
Toll[27], 2010	☺	☺	☺	☹	☹	☺	☺
Panarelli[28], 2012	?	☺	☺	☹	☹	☺	☺
Rockacy[29], 2013	?	☺	☺	☹	☹	☺	☺
Nikiforova[30], 2013	☹	☺	☺	☹	☹	☺	☺
Al-Haddad[31], 2014	☺	☺	☺	☹	☹	☺	☺
Singhi[9], 2014	☹	☺	☺	☹	☹	☺	☺
Kung[32], 2014	☹	☺	☺	☹	☹	☺	☺
Winner[33], 2015	☹	☺	☺	☹	☹	☺	☺
Jones[34], 2015	☺	☺	☺	☹	☹	☺	☺
Singhi[35], 2016	☺	☺	☺	☹	☹	☺	☺
Kadayifci[5], 2016	☺	☺	☺	☺	☹	☺	☺
Singhi[7], 2017	☺	☺	☺	☺	☹	☺	☺

A ☺Low Risk, ☹High Risk, ? Unclear Risk



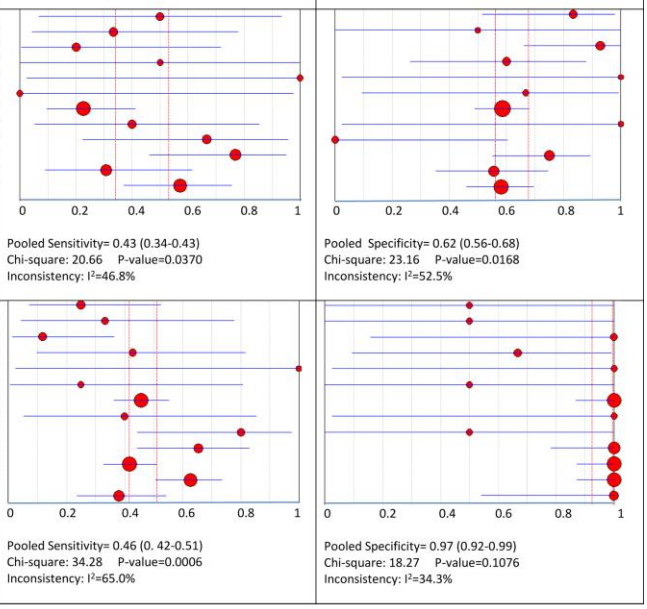
B QUADAS - Risk of Bias QUADAS - Applicability

All studies included in this review showed a "low-risk" classification, as the index test (*KRAS* mutation analysis) and the reference standard (surgical pathology specimen) were reliable and mentioned in all studies. However, a "high-risk" of selection of bias was demonstrated in patient and in flow and timing because only a small proportion of the patients evaluated in all studies, except one, were included in the analysis. In fact, many patients were excluded in all studies as the inclusion criteria requiring surgical pathology as diagnostic reference was not met. Applicability concerns regarding patient selection were also significant in all studies, because the subgroup of PCNs referred for surgery is more often malignant than for patients with pancreatic cysts on clinical surveillance, which would also be targeted with this review.

***KRAS* mutation**

Fourteen articles were included in the meta-analysis for diagnostic accuracy of *KRAS* mutation. For each of the two definitions of relevant cyst, forest plots of sensitivity and specificity with heterogeneity denoted are shown in Figure 3.

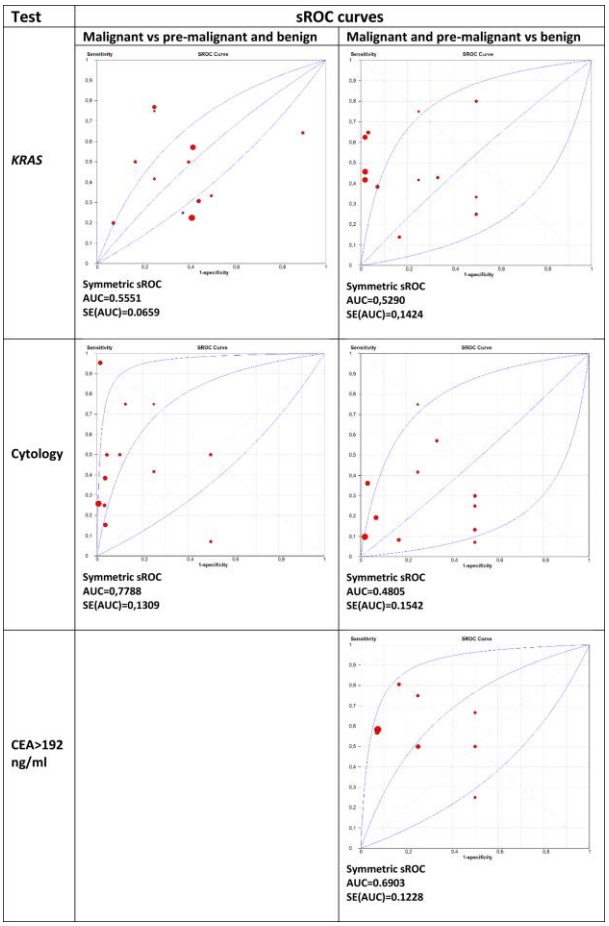
Subgroup	Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Malignant	Shoedel et al. 2006	2	2	2	10	0.50 (0.07-0.93)	0.89 (0.52-0.98)
	Sreenarasimhaih et a	2	0	4	0	0.33 (0.04-0.78)	0.50 (0.00-1.00)
	Sawhney et al. 2009	1	1	4	13	0.20 (0.01-0.72)	0.93 (0.66-1.00)
	Mertz et al. 2011	0	4	0	6	0.50 (0.00-1.00)	0.60 (0.26-0.88)
	Toll et al. 2010	1	0	0	1	1.00 (0.03-1.00)	1.00 (0.03-1.00)
	Panarelli et al. 2012	0	1	1	2	0.00 (0.00-0.98)	0.67 (0.09-0.99)
	Nikiforova et al. 2013	7	46	24	65	0.23 (0.10-0.41)	0.59 (0.49-0.68)
	Kung et al. 2014	2	0	3	1	0.40 (0.05-0.85)	1.00 (0.03-1.00)
	Jones et al. 2015	4	4	2	0	0.67 (0.22-0.96)	0.00 (0.00-0.60)
	Singhi et al. 2016	10	7	3	21	0.77 (0.46-0.95)	0.75 (0.55-0.89)
	Singhi et al. 2017	16	31	12	43	0.57 (0.37-0.76)	0.58 (0.46-0.69)
	Winner et al. 2015	4	12	9	15	0.31 (0.09-0.61)	0.56 (0.35-0.75)
Malignant + Mucinous	Shoedel et al. 2006	4	0	12	0	0.25 (0.07-0.52)	0.50 (0.00-1.00)
	Sreenarasimhaih	2	0	4	0	0.33 (0.04-0.78)	0.50 (0.00-1.00)
	Sawhney et al. 2009	2	0	15	2	0.12 (0.01-0.36)	1.00 (0.16-1.00)
	Mertz et al. 2011	3	1	4	2	0.43 (0.10-0.82)	0.67 (0.09-0.99)
	Toll et al. 2010	1	0	0	1	1.00 (0.03-1.00)	1.00 (0.03-1.00)
	Panarelli et al. 2012	1	0	3	0	0.25 (0.01-0.81)	0.50 (0.00-1.00
	Nikiforova et al. 2013	53	0	63	26	0.46 (0.36-0.55)	1.00 (0.87-1.00)
	Kung et al. 2014	2	0	3	1	0.40 (0.05-0.85)	1.00 (0.03-1.00)
	Jones et al. 2015	8	0	2	0	0.80 (0.44-0.97)	0.50 (0.00-1.00)
	Singhi et al. 2016	17	0	9	15	0.65 (0.44-0.83)	1.00 (0.78-1.00)
	Singhi et al. 2017	47	0	28	27	0.63 (0.51-0.74)	1.00 (0.87-1.00)
	Al-Haddad et al. 2014	16	0	26	6	0.38 (0.24-0.54)	1.00 (0.54-1.00)
	Kadayifci et al. 2016	50	0	70	27	0.42 (0.33-0.51)	1.00 (0.87-1.00)



The definitions of malignant and significant cysts resulted in a different range of specificity and sensitivity of the studies included. In the first case both sensitivity and specificity varied from 0 to 100% and in the second case sensitivity varied from 12% to 100% and specificity from 50% to 100%. In the first subgroup the wide range of sensitivity was largely due to chance variation because of small numbers of patients with the target condition (proven malignant cysts) in the different studies (median, 6; range, 1-31). For instance, if there was only one patient with a proven malignant cyst in a study, and this patient had a positive test, the sensitivity would be 100%, but if he/she had a negative test result, the sensitivity would be 0%. Small numbers of patients with non-malignant cysts in some studies (median, 10; range 1-111) also led to a wide range of specificity.

For each of the two subgroups there occurred a moderate heterogeneity in sensitivity (I^2 = 46.8% vs I^2 = 65.0%) and specificity (I^2 = 52.5% vs I^2 = 34.3%) and therefore random effect models were used. In malignant cysts the pooled sensitivity was 0.43 (95% CI, 0.34-0.53) and the pooled specificity was 0.62 (95% CI, 0.56-0.68). In significant cysts the sensitivity was 0.46 (95% CI, 0.42-0.51) with a specificity of 0.97 (95% CI, 0.92-0.99).

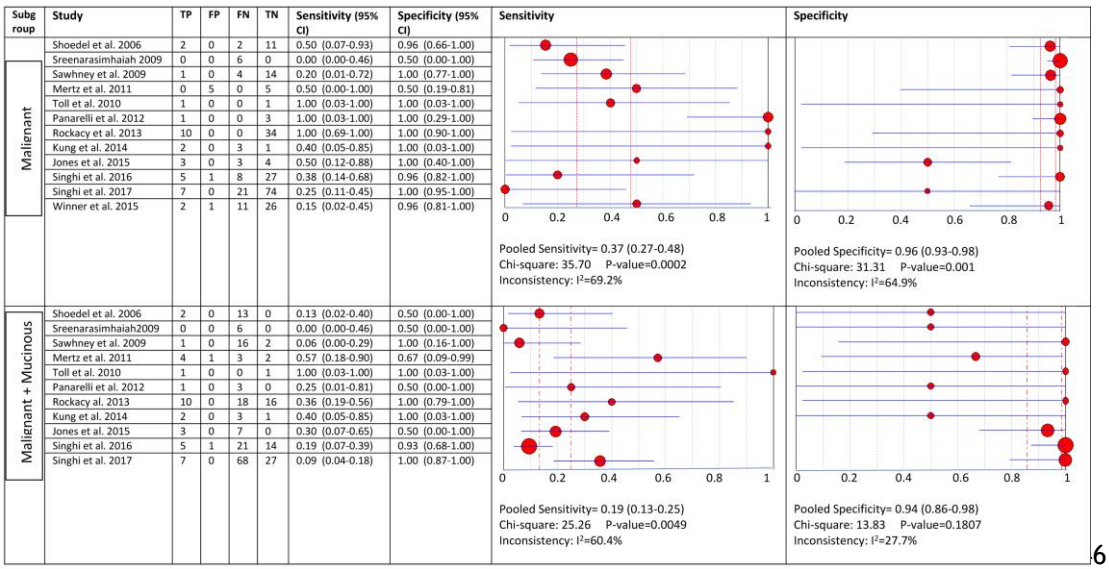
Figure 4 displays the sROC curves of *KRAS* analysis, showing the sensitivity of the individual articles mapped on the vertical scale, 1-specificity on the horizontal scale, summary (sensitivity, 1-specificity) point marked, as well as the sROC curve and the confidence region for the summary (sensitivity, 1-specificity) points. The area under the sROC curve ± SE was 0.5551 ± 0.0659) in malignant cysts and 0.5290 ± 0.1424 in significant cysts. The results of the studies had greater variation in malignant cysts, as shown by the wide confidence region.



The median prevalence of malignant cysts and significant cysts was 29.6% and 82.5% respectively; range, 9.1% - 85.7% and 50% - 100%, respectively. This prevalence was based on the proportion of proven malignant and proven significant cysts in the studies.

Cytology

Twelve articles were included in the meta-analysis for diagnostic accuracy of cytology. Figure 5 shows the forest plots of sensitivity and specificity for the two defined subgroups of cysts.



The forest plots for cytology show variable sensitivities within the papers, from 0-1, which can be due to the small numbers of patients with the target condition in some studies.

In the malignant and significant cysts groups, respectively, there were six and two studies, respectively that recorded sensitivity of cytology as scoring greater than or equal to 0.5.

For each of the two subgroups there existed heterogeneity in sensitivity (I^2 = 69.2% vs I^2 = 60.4%) and specificity (I^2 = 64.9% vs I^2 = 27.7%), and therefore random effects models were used. In malignant cysts the pooled sensitivity was 0.37 (95% CI, 0.27-0.48) and the pooled specificity was 0.96 (95% CI, 0.93-0.98). In significant cysts, the sensitivity was 0.19 (95% CI, 0.13-0.25) with a specificity of 0.94 (95% CI, 0.86-0.98).

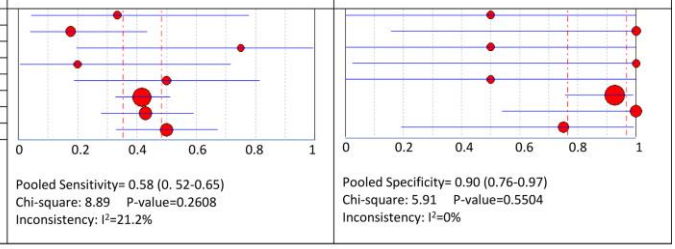
The results were plotted as a symmetrical sROC curve (Figure 4). The area under the sROC curve ± SE was 0.7788 ± 0.1309 in malignant and 0.4805 ± 0.1542 in significant cysts.

The median prevalence of malignant and significant cysts was 29.4% and 86.4%, respectively; range, 9.1% - 85.7% and 50% - 100%, respectively.

CEA >192 ng/ml

Eight articles were included in the meta-analysis for diagnostic accuracy of CEA. Since only four articles (with few patients) allowed the evaluation of accuracy of CEA >192 for diagnosis of malignant cysts, we restricted the analysis to significant cysts. Figure 6 shows the forest plots of sensitivity and specificity.

Subgroup	Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity(95% CI)
Malignant + Mucinous	Sreenarasimhaiah.2009	4	0	2	0	0.67 (0.22-0.96)	0.50 (0.00-1.00)
	Sawhney et al. 2009	14	0	3	2	0.82 (0.57-0.96)	1.00 (0.16-1.00)
	Panarelli et al. 2012	1	0	3	0	0.25 (0.01-0.81)	0.50 (0.00-1.00)
	Kung et al. 2014	4	0	1	1	0.80 (0.28-0.99)	1.00 (0.03-1.00)
	Jones et al. 2015	5	0	5	0	0.50 (0.19-0.81)	0.50 (0.00-1.00)
	Kadayifci et al. 2016	70	2	50	25	0.58 (0.49-0.67)	0.93 (0.76-0.99)
	Al-Haddad et al. 2014	24	0	18	6	0.57 (0.41-0.72)	1.00 (0.54-1.00)
	Winner et al. 2015	18	1	18	3	0.50 (0.33-0.67)	0.75 (0.19-0.99)



The forest plots for cytology showed a sensitivity from 0.5 to 0.82 and a specificity from 0.5 to 1.

There existed homogeneity in sensitivity (I^2 = 21.2%) and specificity (I^2 = 0%). The pooled sensitivity was 0.58 (95% CI, 0.52-0.65) and the pooled specificity was 0.90 (95% CI, 0.76-0.97).

The area under the sROC curve ± SE was 0.6903 ± 0.1228.

The median prevalence of significant cysts was 89.7%; range 81.6% - 100%.

Publication bias

Regression analyses of funnel plots were not statistically significant (p>0.05), suggesting that publication bias was not a major determinant.

4. DISCUSSION

In this systematic review and meta-analysis we performed a comparative analysis of the current standard tests in PCF obtained by EUS-FNA (CEA and cytology) and molecular analysis (*KRAS* mutation) in PCNs. The comparative analysis included all studies, evaluating the three tests separately.

Our meta-analysis is the largest published, and included 731 patients, all with molecular analysis performed by NGS pre-operatively, and all patients with a surgical pathology specimen as reference standard for diagnosis. We analyzed these three markers, for diagnosis of significant as compared to benign cysts and for diagnosis of malignant versus non-malignant cysts, because relevant clinical decisions apply to these categories.

The comparative analysis of *KRAS*, cytology, and CEA for cyst diagnosis, showed that cytology alone had the highest accuracy (AUC = 0.7788) for the diagnosis of malignant cysts, and CEA the highest accuracy (AUC = 0.6903) for the diagnosis of significant cysts. *KRAS* mutational analysis had the worst performance for both groups of lesions with AUC = 0.551 for malignant and AUC = 0.46 for significant cysts. The specificity of *KRAS* for diagnosis of significant cysts was high (97%) which makes it useful to diagnose these lesions, but due to low sensitivity (46%), *KRAS* should not be used to exclude the diagnosis, as false negative results are common. Similar results for *KRAS* were previously published by Guo et al,[36] who analyzed several molecular tests for improving differential diagnosis of PCNs.

As DNA testing continues to evolve, questions remain about its accuracy, how it influences patient management, and in what order it should be performed to better support clinical decisions. Previous studies[6] have shown that DNA testing combined with clinical features increased correct PCNs diagnosis compared to either one. With the multiple recent advances in biomarkers, particularly DNA-based mutations, molecular genetics will probably prove to be useful in management of PCNs.[37] In a previous meta-analysis, cytology in pre-operative diagnosis of PCNs has shown low sensitivity for diagnosis,[38] recommending additional tests to improve diagnosis. Another published meta-analysis evaluating diagnostic accuracy of EUS-FNA with CEA and cytology in differentiating mucinous cysts has demonstrated to be accurate to confirm the diagnosis but performs poorly in excluding it.[39] The role of *KRAS* as individual screening test has also been analyzed before[40] with poor accuracy and added benefit coming from a combined approach with cytology. Finally, a recently published meta-analysis supporting *KRAS*, *GNAS,* and *RNF43* mutations as diagnostic markers of IPMNs[41] used different methods for mutation detection, different tumor materials, and clinicopathologic data as reference standard for diagnosis, which may limit its clinical application in pancreatic cystic lesions, in which mutational analysis is performed solely in cystic fluid.

In our study the pooled sensitivities of *KRAS*, cytology, and CEA besides being limited, also varied considerably. On the other hand, specificity was uniformly high for the tests analyzed, particularly for *KRAS* and CEA for diagnosis of significant cysts and cytology for both malignant and significant cysts.

By estimating the pooled sensitivities, we sought to determine which of the tests had a better performance.

For a group of 100 patients with a pancreatic cyst and a prevalence of malignant cysts of 30%, the presence of a *KRAS* mutation would diagnose 13 (TP), miss 17 (FN), and 27 (FP) would be unnecessarily operated. For a prevalence of significant cysts of 86%, 40 would be correctly diagnosed (TP), 46 would be missed by *KRAS* (FN), and none would be unnecessarily referred for surgery/surveillance (FP).

With respect to cytology, a positive result for the diagnosis of malignant cysts in a group of 100 patients with a prevalence of 30% of malignant cysts would diagnose 11 (TP), would miss 19 (FN) patients, and 3 (FP) would be unnecessarily referred for surgery. For significant cysts, with a prevalence of 86%, a positive cytology would diagnose 16 (TP) PCNs, would miss 70 (FN) PCNs, and none (FP) would be unnecessarily referred for surgery/surveillance.

If 100 patients with PCNs evaluated with a CEA >192ng/ml in PCF and a prevalence of significant cysts of 86%, 52 (TP) would be diagnosed by CEA and 36 (FN) would be missed by the test, with 1 (FP) that would be unnecessarily referred for surgery/follow-up.

Although both *KRAS* and CEA are useful for mucinous cyst diagnosis that were classified in this meta-analysis as significant, based on our results we can conclude that CEA would miss fewer PCNs (lower FN rate) with only one FP. Concerning malignancy diagnosis, cytology is the best diagnostic test, because although *KRAS* mutation can diagnose more malignant cysts (13 versus 11), it would have significantly

higher numbers of FP diagnosis (27 versus 1). We can conclude that *KRAS* mutation is not better than CEA for significant cyst diagnosis and that cytology is the most accurate test for malignancy diagnosis.

However, we should remember that in routine clinical practice a major pitfall for PCNs diagnosis is the frequently scant volume of PCF obtained, precluding routine PCF testing. As mutation analysis requires less volume of PCF, it may be an alternative test in these circumstances. This major advantage of molecular analysis was not possible to evaluate because the volume of cystic fluid obtained was not available in most studies analyzed.

Additionally, combining *KRAS* mutation with conventional testing increased the sensitivity of PCNs diagnosis without compromising specificity. We extracted data from the studies analyzed in this meta-analysis to evaluate the added value of *KRAS* in conjunction with cytology and CEA, but the available data were limited to four studies[27,28,32,34] (Table 1), making the analysis inconclusive.

The strengths of our work are the use of strict exclusion criteria, with all analyzed patients with an analyzed surgical pathology as the reference standard and avoiding bias related to methodological limitations of the studies evaluated. We chose to include only patients with a surgical pathology as the reference standard because histopathology is the gold standard for diagnosis of neoplasia. This is an important strength of our systematic review and provides a more realistic and accurate estimate for the index and comparative tests evaluated. In previous studies of accuracy of cytology including both surgical pathology and clinical follow-up[39] as reference standard, pooled sensitivities were 12% higher than in studies with exclusive surgical pathology[40] as reference standard in the diagnosis of mucinous cysts, with overestimation of test accuracy.

Limitations of this study include incomplete reporting in diagnostic test accuracy in primary studies, with no separate information for distinction of malignant and mucinous cysts in two studies[5,31] and in another two for distinction of benign and pre-malignant mucinous cysts.[33,29] These four studies were included in the group of seven studies with more patients analyzed in the meta-analysis. Another limitation is the time elapsed between the index tests and the reference standard. The final diagnosis could have been made at different time intervals from the tests. If the time between index tests and reference standard is too long, the true diseased status of the patient may have changed by the time the reference standard was assessed. Finally, the low number of malignant cysts per study (0 to 13), except for four studies[30,9,5,7] may contribute to part of the heterogeneity in the sensitivity observed.

Future perspectives

With the increasing diagnosis of asymptomatic PCNs, some with malignant potential, there is a growing need to find accurate biomarkers of malignancy in these lesions, to reduce surgeries on benign cysts and still diagnose and resect early malignant lesions with favorable prognosis. DNA molecular markers, particularly *KRAS* mutation, which is an early event in pancreatic carcinogenesis, has the potential to fulfill this need, but clinicians should be aware of their current limitations in diagnostic performance and type of lesions identified.

Certainly, the significant costs, logistic difficulties in collecting and preserving material for future molecular analysis in busy general hospitals, and the technical complexity of the test, make its generalized use difficult in clinical practice. Moreover, large multicenter validation studies are still missing.

Additionally, there is a need for more trials to confirm their clinical relevance in patient outcomes, such as early cancer diagnosis, number of surgeries of benign lesions avoided, and prognostic value in numerous cysts that require periodic surveillance.

Moreover, for successful massive implementation of molecular markers in pancreatic cyst clinics, a validation of *KRAS* mutation as a complementary test to patients with an unavailable CEA level and a non-diagnostic cytology will be insufficient. Its development as a universal, highly accurate, first line test with clinical impact in cyst diagnosis and patient management will be required. NGS reliably allows analysis of multiple gene panels both in PCF and peripheral blood and offers an attractive option to increase the accuracy of molecular analysis in diagnosis and risk stratification of these lesions.[42]

Finally, with current evidence, *KRAS* can only be recommended as a second line test in the case that CEA and cytology of PCF are non-diagnostic. It would be useful to determine the additional value of the *KRAS* in combination with the other tests and to evaluate the adequate order of the tests, in order to maximize the diagnoses of malignant and/or significant cysts.

Conclusion

The intended use and clinical role of *KRAS* mutational analysis in the present should be limited to patients with an undefined CEA level and a non-diagnostic cytology, serving only as a complementary diagnostic test due to its limited accuracy. *KRAS* has lower diagnostic accuracy than CEA and cytology and should not replace standard EUS-FNA analysis. Clinicians should be aware of a significant rate of false positive results of *KRAS* mutation if the diagnosis of a malignant cyst is under consideration.

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1.2. Genetic testing *vs* microforceps biopsy in pancreatic cysts: Systematic review and meta-analysis. Published in WJG.

Abstract

BACKGROUND

Carcinoembryonic antigen (CEA) and cytology in pancreatic cystic fluid are suboptimal for evaluation of pancreatic cystic neoplasms. Genetic testing and microforceps biopsy are promising tools for pre-operative diagnostic improvement but comparative performance of both methods is unknown.

AIM

To compare the accuracy of genetic testing and microforceps biopsy in pancreatic cysts referred for surgery.

METHODS

We performed a literature search in Medline, Scopus, and Web of Science for studies evaluating genetic testing of cystic fluid and microforceps biopsy of pancreatic cysts, with endoscopic ultrasound with fine-needle aspiration (EUS-FNA) prior to surgery and surgical pathology as reference standard for diagnosis. We evaluated the diagnostic accuracy for: 1- benign cysts; 2- mucinous low-risk cysts; 3- high-risk cysts, and the diagnostic yield and rate of correctly identified cysts with microforceps biopsy and molecular analysis. We also assessed publication bias, heterogeneity, and study quality.

RESULTS

Eight studies, including 1206 patients, of which 203 (17%) referred for surgery who met the inclusion criteria were analyzed in the systematic review, and seven studies were included in the meta-analysis. Genetic testing and microforceps biopsies were identical for diagnosis of benign cysts. Molecular analysis was superior for diagnosis of both low and high-risk mucinous cysts, with sensitivities of 0.89 (95%CI: 0.79-0.95) and 0.57 (95%CI: 0.42-0.71), specificities of 0.88 (95%CI: 0.75-0.95) and 0.88 (95%CI: 0.80-0.93) and AUC of 0.9555 and 0.92, respectively. The diagnostic yield was higher in microforceps biopsies than in genetic analysis (0.73 vs 0.54, respectively) but the rates of correctly identified cysts were identical (0.73 with 95%CI: 0.62-0.82 vs 0.71 with 95%CI: 0.49-0.86, respectively).

CONCLUSION

Genetic testing and microforceps biopsies are useful second tests, with identical results in benign pancreatic cysts. Genetic analysis performs better for low- and high-risk cysts but has lower diagnostic yield.

Key words

Pancreatic cysts; Endoscopic ultrasound; Endoscopic ultrasound with fine-needle aspiration; Genetic testing; Microforceps biopsy; Molecular analysis; KRAS; Carcinoembryonic antigen; Cytology

Text

INTRODUCTION

Pancreatic cystic neoplasms (PCNs) are on the rise in clinics due to an ageing population and the increase in routine use of high-quality abdominal imaging[79]. PCNs are generally classified into two main groups: mucinous cystic neoplasms (MCNs) and non-mucinous cystic neoplasms (NMCN). MCNs include intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystadenomas, which are precursor lesions of pancreatic carcinoma, and may be low-risk (pre-malignant with low or intermediate-grade

atypia) or high-risk: pre-malignant with high-grade atypia (HGA) or malignant, including adenocarcinomas secondarily cystic. NMCNs include serous cystadenomas and inflammatory cysts (pseudocysts), mostly benign cysts, but may include some rare lesions, considered high-risk as cystic neuroendocrine tumors (cNETs), and acinar cell cystadenomas (ACCs). The heterogeneity in malignant potential, increased frequency, and significant morbidity and mortality of surgical treatment, makes pre-operative diagnosis of PCNs essential for management. The treatment options for PCNs encompass surgery or conservative surveillance for MCNs, according to malignancy risk, or no further evaluation for most NMCNs.

The differentiation between MCNs and NMCNs is critical, because a misdiagnosis of a MCN can lead to a missed opportunity to treat pancreatic cancer in an early stage and a misdiagnosis of NMCN can result in unnecessary surgery or surveillance with associated morbidity, costs, and negative impact on quality of life.

Currently, morphologic characterization of PCNs and pancreatic cystic fluid (PCF) analysis for carcinoembryonic Antigen (CEA) and cytology are central in diagnosis. A CEA level ≥192 ng/mL is the most accurate diagnostic test for MCNs and cytology is highly specific for malignancy[127], but with suboptimal results in large studies with surgical pathology as the gold standard[84]. In fact, a significant part of these lesions remains indeterminate and incorrect pre-operative diagnosis occurs in one third of patients[5,128], making new reliable diagnostic tools urgently needed.

In the last decade numerous studies have shown that genetic analysis of aspirates obtained by EUS-FNA provided a better characterization of PCNs than CEA and cytology[19-26]. Next-generation sequencing (NGS) is a very sensitive technique for detection of genetic mutations that allows the rapid detection of mutations in pre-defined panels of cancer genes, even in samples with limited DNA content, such as PCF. NGS requires storage, infrastructure, data processing, and expert personnel. Moreover, to be cost-effective, large numbers of samples need to be processed, making it applicable only in large centralized laboratories. These reasons make the implementation of NGS in clinical practice still a matter of debate.

The clinical need of better diagnostic tests in PCNs has recently led to the development of a through-the-needle miniature biopsy device for use during EUS-FNA[43,44]. The Moray micro forceps biopsy (MFB) device (US Endoscopy, Mentor, Ohio) is disposable and can pass through a standard 19-gauge EUS-FNA needle that is already used routinely. It allows tissue sampling from the cyst wall, septa or mural nodules and the obtention of a histological evaluation of the epithelial architecture and subepithelial stroma[150]. Adding to the high technical success and excellent safety profile[151,47], the new device has shown to improve the diagnostic accuracy of specific cyst subtypes[46,45]. Another major advantage of MFB is the simultaneous tissue sampling and PCF acquisition, with just an additional histologic analysis that follows standard definitions and is already routine in clinics.

The aim of this systematic review and meta-analysis is to evaluate the diagnostic performance of molecular analysis (MA) and MFB and find the most robust additional diagnostic technique in PCNs, in the pre-operative setting.

MATERIALS AND METHODS

This systematic review and meta-analysis is conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses of Diagnostic Test Accuracy Studies, the PRISMA-DTA Statement[152], and the protocol is registered at PROSPERO (CRD42018111910).

Literature search and study selection

A comprehensive search of databases, including Medline, Scopus, and Web of Science, for the past 8 years (January 1st, 2010 to July 31st, 2018) and restricted to human studies was performed. No language restrictions were applied. The following search terms were used in two independent searches: "pancreas", "cyst", "molecular", "analysis"; and "micro", "forceps", "microforceps", "biopsy". A search of related articles was performed, adding additional studies. Duplicate articles, reviews, trials including other kinds of neoplasms, and trials with molecular markers not compliant with the defined inclusion criteria were removed. The references of all selected studies were hand-searched for additional articles.

Inclusion criteria: Published studies were included in the meta-analysis if they analyzed: (1) patients with symptomatic or incidental pancreatic cysts with a definitive surgical pathology diagnosis; (2) genetic mutations performed with high sensitive techniques, such as NGS in PCF obtained by EUS-FNA prior to surgery; (3) at least four genetic mutations, including *KRAS, GNAS, VHL*, and at least another genetic mutation representative of aggressive neoplasms (*PIK3CA, TP53, SMAD4, PTEN, CDKN2A*); (4) PCNs evaluated by EUS-FNA with MFB for diagnosis; and (5) surgical pathology specimens with available data.

Exclusion criteria: (1) Studies of MA with fewer than the four genetic mutations previously defined; (2) studies involving solid pancreatic lesions; (3) studies using PCF not obtained by EUS-FNA; (4) reviews, case reports, case series with fewer than five patients, letters to editor, exploratory studies, and papers published only in abstract form; (5) studies with cytology and clinical surveillance as standard of diagnosis. Two authors (SF and AL) independently judged study eligibility and disagreements were resolved by consensus.

Histological criteria: We classified the PCNs of the included studies into three main groups: (1) high-risk cysts (adenocarcinoma or high grade dysplasia in IPMNs and MCNs, secondarily cystic adenocarcinomas, cNETs, and ACCs; (2) low-risk mucinous cysts (IPMNs and MCNs with intermediate or low-grade dysplasia); and (3) benign cysts (SCAs, pseudocysts, and other rare cysts (RCs) included in some articles, as retention cysts, lymphoepithelial cysts, epidermoid cysts, squamoid cysts).

Tests under investigation: The index tests were: (1) MA of PCF; and (2) MFB of PCNs, including cyst wall, septs, and nodules. A diagnosis of cNET or ACC does not warrant a malignancy diagnosis, but surgery is recommended in surgically fit patients. Due to a recommendation of identical treatment to malignant and mucinous high-risk cysts, for the purpose of analysis in this study, each one of these diagnoses was classified as a high-risk cyst.

Data extraction

After study selection, two authors (SF and AL) extracted and registered the data from each study onto a standardized worksheet. Disagreements were discussed and reviewed by a third author (LP). The data retrieved were: first author, publication year, study period and design (prospective or retrospective), reference for diagnosis, sample size (all patients included in the study), technical success, adverse events, diagnostic yield, surgical cohort (number of patients with a surgical pathology specimen), cyst size, cyst location, specific cyst types, number of high-risk cysts, mucinous low-risk and benign cysts

diagnosed by MA and MFB comparing to surgical pathology specimens. In the MFB studies, technical success was defined as the ability to puncture the cysts and perform the biopsies; and the diagnostic yield was defined as the ratio between the number of patients included in the study and the patients in whom enough material allowed the acquisition of a histopathologic diagnosis. In the MA group, diagnostic yield was defined as a ratio between the number of patients included in the study and the number of patients with DNA available to perform molecular analysis in PCF.

Outcomes

The primary outcomes of this study were the data to obtain the accuracies of MA and MFB for the diagnosis of PCNs, including high-risk cysts, mucinous low-risk cysts, and benign cysts. Secondary outcomes were the diagnostic yield of genetic testing and MFB and the number of cysts correctly identified for each of the tests studied.

Quality analysis

Methodological quality of included primary studies was assessed by two authors (SF and AL) using the modified QUADAS-2 tool[131]. The PRISMA-DTA Statement recommendations were used for reporting this systematic review[22,130].

Statistical analysis and data synthesis

The reference standard was a surgical pathology specimen that allowed the classification of PCNs into three defined groups of diagnosis: high-risk cysts, mucinous low-risk cysts, and benign cysts. This resulted in a two-by-three table with correct and incorrect test results in each of the three referenced groups, for each of the tests analyzed, MA and histology were obtained by MFB.

To calculate tests' accuracy and to reflect on the categories that are useful in clinical practice and that guide management, we constructed two-by-two tables, considering three definitions of "relevant" cysts: (1) High-risk cysts – proven malignant cysts, IPMNs, and MCNs with HGA, cNETs, ACCs; Non-High-risk cysts – all cysts except those proven to be high-risk. (2) Low-risk mucinous cysts – proven mucinous low-risk cysts; High-risk cysts – all except those proven to be mucinous low-risk or benign. And (3) Non-benign cysts – all cysts except those proven to be benign; Benign cysts – proven benign cysts.

The ability of the tests to discriminate "relevant" and "non-relevant" cysts using the three definitions of "relevant cysts" was evaluated and the accuracy of the two tests was compared.

The data of the two-by-two tables were used to calculate sensitivity and specificity for each study. We present individual study results graphically by plotting the estimates of sensitivity and specificity (and their 95% confidence intervals (CI)) in both forest plots and on the summary receiver operating characteristic (sROC) curve plots. The area under the curve (AUC) is equal to 1 for a perfect test and 0.5 for a completely uninformative test. The AUC is equal to the probability that if a pair of relevant and non-relevant cysts is selected at random, the relevant cyst will have a higher test result than the non-relevant cyst. Pooled estimates of the sensitivity and specificity were obtained by the DerSimonian-Laird method (random effect model) to incorporate variation among studies, when data are heterogeneous. Otherwise, we used the Mantel-Haenszel method (fixed effect model).

Heterogeneity was investigated in the first instance through visual examination of forest plots of sensitivities and specificities and through visual examination of the ROC plot of the raw data. Last, we used statistical tests, including chi-square and Cochran-Q to evaluate if the differences across the studies were greater than expected by chance alone. A low p-value suggests presence of heterogeneity.

In addition to these statistics we used the statistic I^2 of Higgins, which has been proposed as a measure to quantify the amount of heterogeneity[132,133]. The scale of I^2 has a range of 0 to 100% and values on the order of 25%, 50% and 75% are considered low, moderate, and high heterogeneity, respectively.

Another goal of this work was to obtain, for each of the tests, the correctly identified cyst rate and the diagnostic yield in predicting a histopathologic diagnosis.

We used Comprehensive Meta-Analysis software (Version 2.0) for assessment of diagnostic yield of the tests and Meta-DiSc (version 1.4 – Meta-Analysis of Diagnostic and screening tests[136]) to obtain the accuracy of each of the tests.

RESULTS

Systematic Review

Our search revealed 16 study titles and abstracts for MFB and 264 titles for MA. In Figure 1A and B are described the selection process of the articles included in this study.

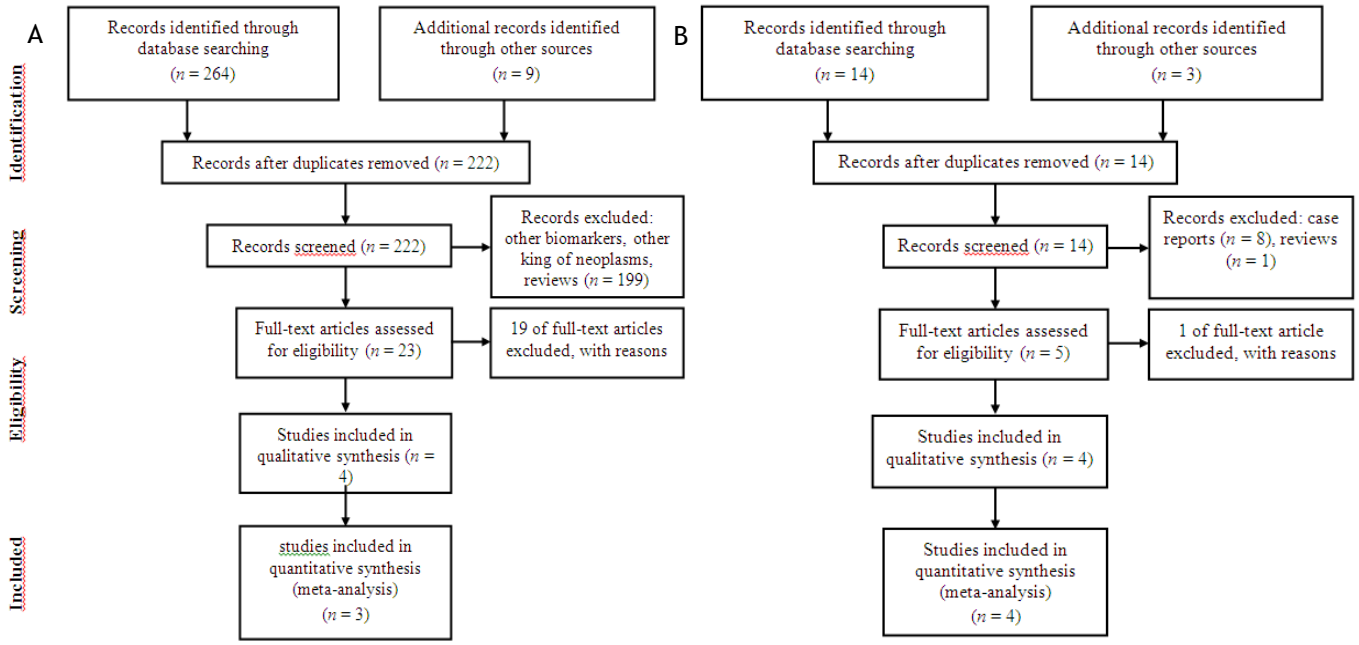


Figure 1 Flowchart with identification of eligible studies. A: Molecular analysis; B: Microforceps biopsy.

After all steps, eight studies were considered suitable for qualitative and seven for quantitative analysis. We excluded 20 full-text articles after review, because they were case series of two patients[44] (*n* = 1), exploratory or pilot studies[153,111] (*n* = 2), no information of mutation status was available[154] (*n* = 1), pancreatic cystic fluid was obtained during surgery[155] (*n* = 1), insufficient or absent data of cysts with surgical pathology diagnoses[24,91,156] (*n* = 3), and mutations only of *KRAS* and/or *GNAS*[26,142-157] (*n* = 12).

Of the eight studies that met the inclusion criteria, design was retrospective in six and prospective in two, all were published from 2015 to 2018. These eight studies included a total of 1206 patients, of which 203 (17%) underwent surgical resection and a surgical pathology specimen was available as reference standard and included in the analysis. We excluded all patients with cytology and clinical follow-up data, but for whom a surgical pathology specimen was not available. The characteristics of the studies, surgical pathology diagnoses, and MA and MFB results are presented in Tables 1[32,45-47] and 2[18-21].

Table 1 Characteristics of studies of molecular analysis included in the analysis

Ref.	Type of study (study period)	Number of genes/DNA quantity, ng/microL(mean and interval)	Diagnosis	Sample size (all patients in the study)	Technical success (successful molecular analysis)	Adverse events (major/minor)	Diagnostic yield (patients with DNA mutations detected)	Surgical cohort	Mean cyst size and range (mm)	Cysts < 3 cm	Cyst location (H/B/T) Cysts with mutations	Specific Cyst Diagnosis (MA/all patients)	Specific Cyst Diagnosis (MA/surgical pathology)	High risk cysts (MA/surgical pathology)	Mucinous low-risk cysts (MA/surgical pathology)	Benign cysts (MA/surgical pathology)	Specific cyst type (MA/surgical pathology)								
																	IPMN-ADC	IPMN-HG	IPMN-LG	MCN-HG	MCN-LG	SCA	Pseudocyst	NET	Other[4]
Jones *et al*[45], 2015	Prospective (Mar 2013-Feb 2014)	39/12.3 (0-1283)	Pathology or cytology	86	79 (only 3 due to NGS failure)	NA	46	10	NA	NA	NA	72/92	5/10	1/6	4/4	0/0	1/4	0/2	4/4	0/0	0/0	0/0	0/0	0/0	0/0
Singhi *et al*[46], 2016	Retrospective (Jan 2014-May 2015)	6/NA	Pathology or clinicopathologic	225	225	NA	118	41	2.8 (8-115)	152	95/81/49	130/225	32/41	10/14	12/13	10/14	9/9[2]	1/2[2]	12/12	0/0	0/1	2/2	8/8	0/2	0/5[3]
Rosenbaum *et al*[32], 2016	Retrospective (Mar 2013-Jun 2015)	9/NA	Pathology or clinicopathologic	105	105	NA	67	25	NA	NA	NA	67/113[1]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Singhi *et al*[47], 2017	Prospective (Jan 2014-Ju 2017)	11/6.93 (0.01-248)	Pathology or cytology	642	595	NA	335	102	2.7 (8-210)	NA	320/B+T=306	357/626	77/102	17/29	41/47	19/26	13/13	4/4[3]	39/39	0/2	2/8	2/3	17/17	0/9	0/7[3]
				1058	1004	NA	566 (54%)	178	2,7(8-210)	152		828/965 (86%)	114/153 (74%)	28/49 (62%)	57/64 (89%)	29/40 (73%)	23/26	5/8	55/55	0/2	2/9	4/5	25/25	0/11	0/12

[1]113 samples of 105 patients; [2]*TP53*, *PIK3CA*, *PTEN*; [3]1 ACC; [4]includes: ACC, retention cyst, lymphoepithelial cyst, epidermoid cyst, and squamoid cyst. MA: Molecular analysis; NA: Non-available data; ACC: Acinar cell cyst; H/B/T: Head body and tail of the pancreas; ADC: Adenocarcinoma; HG: High-grade; LG: Low-grade; NET: Neuroendocrine tumor.

Table 2 Characteristics of studies of microforceps biopsies included in the analysis

Ref.	Type of study (study period)	Diagnosis	Sample size (all patients in the study)	Technical success (successful cyst puncture and biopsy)	Adverse events (major/ minor)	Diagnostic yield (histopathologic diagnosis availabl e)	Surgical cohort	Mean cyst size and range, (mm)	Cysts < 3 cm	Cyst location (H/B /T)	Specific cyst diagnosis (MFB /all patients)	Specific cyst (MFB/surgical pathology)	High risk cysts (MFB/surgical pathology)	Mucinous low risk cysts (MFB/surgical pathology)	Benign cysts (MFB /surgical pathology)	Specific cyst type (MFB/surgical pathology)								
																IPMN-ADC	IPMN-HG	IPMN-LG	MCN-HG	MCN-LG	SCA	Pseudocyst	NET	Other (ACC)
Mittal *et al*[18], 2018	Retrospective (Dec 2016-June 2017)	Pathology, cytology and cyst fluid analysis	27	27	0/0	24	4	38 (15-70)	20	14/B+T=13	24/27	2/4	1/1	1/3	0/0	0/0	0/0	1[2]/3	-	NA	-	-	1/1	-
Zhang *et al*[20], 2018	Retrospective (Jan 2016-Set 2017)	Pathology, cytology and cyst fluid analysis	48	46	0/0	36	10	31 (12-60)	23	13/22/13	24/48	7/10	3/4	4/5	1/1	1[1]/3	1[3]/0	4/4	-	0[1]/1	1/1	-	-	1/1
Basar *et al*[21], 2018	Retrospective (2015-2016)	Pathology, cytology and cyst fluid analysis	42	38	0/2	26	7	28 (12-60)	24	16/17/9	15/42	6/7	4/4	1/2	1/1	2/2	-	1/1	-	0[1]/1	1/1	-	1/1	1/1
Kovacevic *et al*[19], 2018	Retrospective (Sep 2016-Oct 2017)	Pathology or multidisciplinary conference	31	27	1/2	22	4	34 (12-130)	14	NA/NA/NA	24[4]/31	3/4	0/1	2/2	1/1	0/1	-	3[3]/2	-	-	-	1/1	-	-
			148	138	1/4	108/148 (73%)	25	33 (12-130)	81		63/148 (42%)	18/25 (72%)	8/10 (80%)	8/12 (67%)	3/3 (100%)	3/6	1/0	8/10	-	0/2	2/2	1/1	2/2	2/2

[1]1 nondiagnostic on MFB; [2]2 nondiagnostic on MFB; [3]1 false negative IPMN associated invasive carcinoma; [4]2 cases with a suspicion of pseudocyst. MFB: Microforceps biopsy; NA: Non-available data; ND: Nondiagnostic; ACC: Acinar cystadenoma; H/B/T: Head body and tail of the pancreas; ADC: Adenocarcinoma; HG: High-grade; LG: Low-grade; NET: Neuroendocrine tumor.

Quality assessment and publication bias: Methodological quality of primary studies included was assessed by two authors (SF and AL) using the modified QUADAS-2 tool[131], which evaluates the quality of articles for systematic reviews of diagnostic accuracy studies in four domains, including patient selection, index test, reference standard, and flow and timing, for risk of bias and applicability concerns. Results are presented in Figure 2, which was sketched with templates available at www.quadas.org.

A

Study	Risk of bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Jones *et al*[145], 2015	☺	☺	☺	☹	☹	☺	☺
Singhi *et al*[30], 2016	☺	☺	☺	☹	☹	☺	☺
Rosenbaum *et al*[91], 2016	☹	☺	☺	☺	☹	☺	☺
Singhi *et al*[113], 2017	☺	☺	☺	☺	☹	☺	☺
Mittal *et al*[151], 2018	☹	☺	☺	☺	☹	☺	☺
Zhang *et al*[46], 2018	☹	☺	☺	☺	☹	☺	☺
Basar *et al*[45], 2018	☹	☺	☺	☺	☹	☺	☺
Kovacevic[47], 2018	☹	☺	☺	☺	☹	☺	☺

☺Low risk; ☹High risk; ? Unclear risk.

B

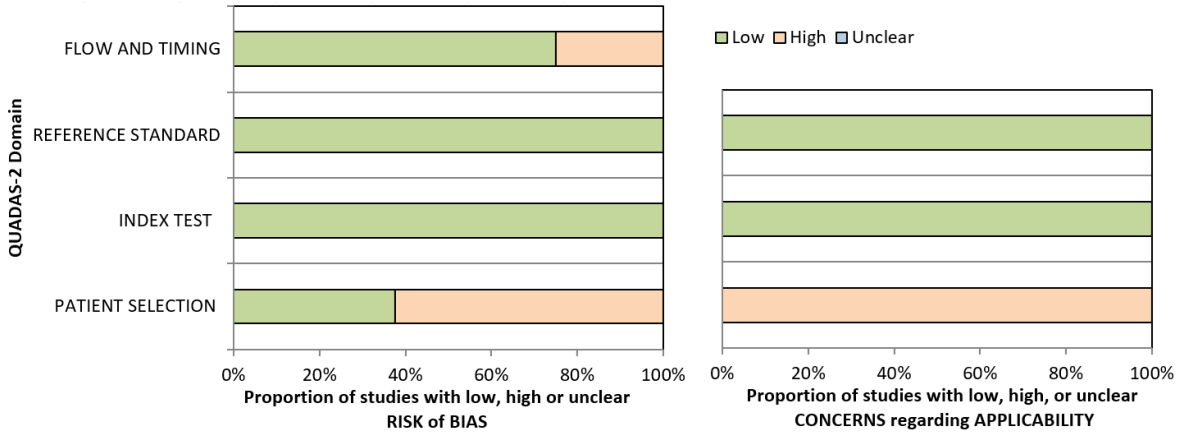


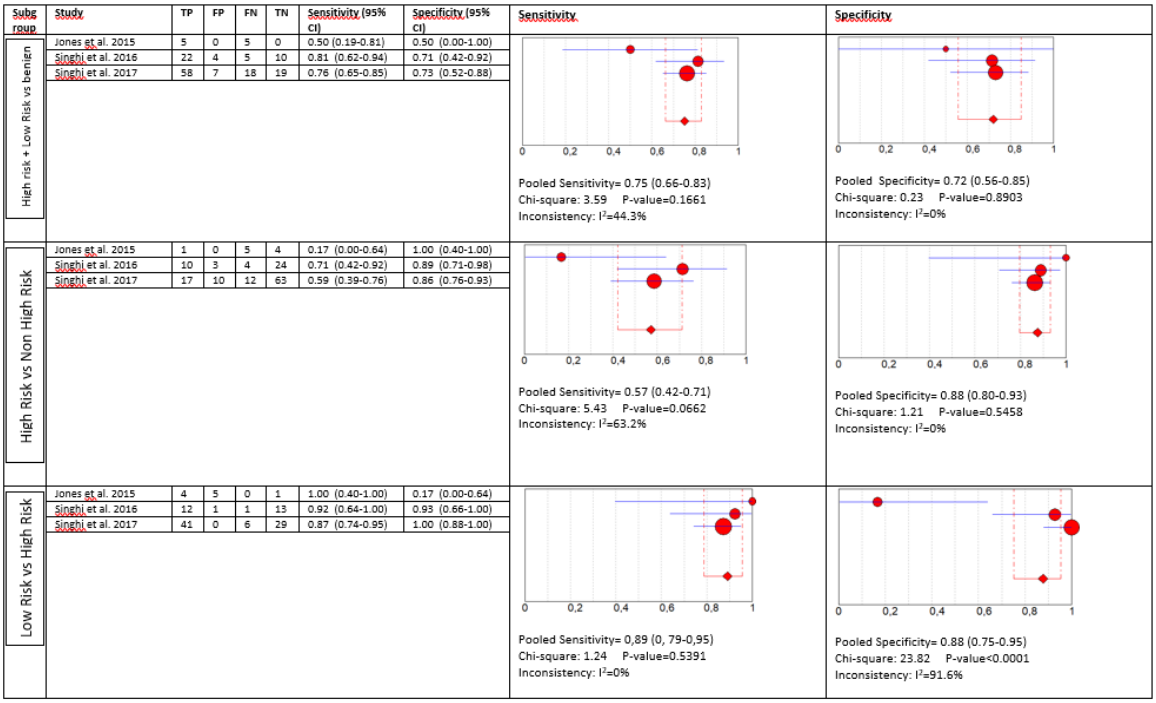
Figure 2 Quality assessment of the studies using QUADAS-2. A: Tabular presentation of risk bias for each study; B: Graphical display of bias.

The studies included in this review all showed a "low-risk" classification as the index tests (MA and MFB) and the reference standard (surgical pathology specimen) were reliable and mentioned in all studies. However, a "high-risk" of selection bias was demonstrated in patient selection (neither random nor sequential patients included in several studies) and in flow and timing because only a small proportion of the patients evaluated in all studies, except one, were included in the analysis. In fact, most patients were excluded in all studies as the inclusion criteria requiring surgical pathology as diagnostic reference were not met. Applicability concerns in patient selection were also significant in all studies, because the subgroup of PCNs referred for surgery is more often malignant than PCNs on surveillance, which

would also be targeted with this review. Because of this bias, there may be an overestimation of both the sensitivity of the index tests, due to a more severe spectrum of PCNs that are referred for surgery, and the positive predictive value (PPV) for diagnosis of high-risk cysts, due to an increased prevalence of malignant cysts in a surgical cohort of PCNs.

Meta-analysis

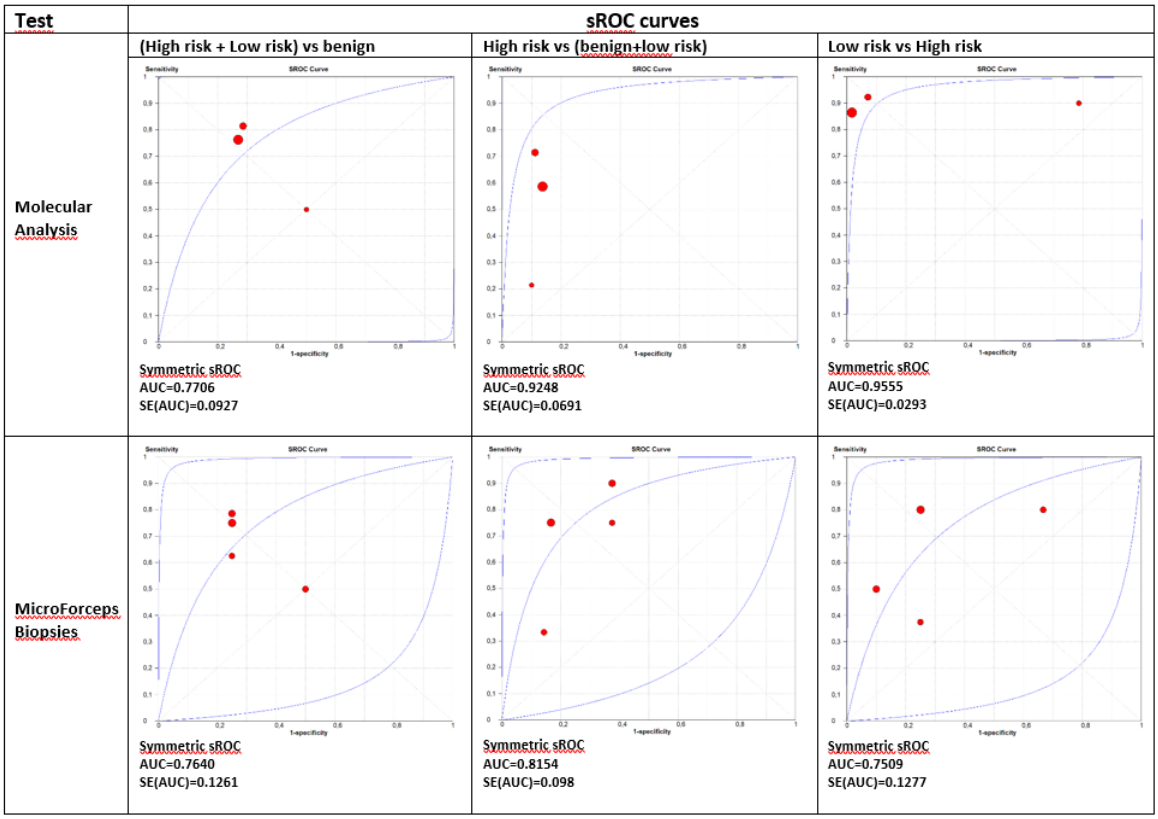
Molecular analysis: Four articles were included in the meta-analysis for diagnostic accuracy of MA. For each of the three definitions of relevant cyst, forest plots of sensitivity and specificity with heterogeneous denoted are shown in Figure 3.



0.66-0.83) and the pooled specificity was 0.72 (95%CI: 0.56-0.85) for MA. In the subgroup of high-risk cysts that require surgery, comparing to other cysts requiring conservative management, the sensitivity was 0.57 (95%CI: 0.42-0.71) with a specificity of 0.88 (95%CI: 0.80-0.93). In the subgroup of low-risk mucinous cysts comparing to high-risk, the pooled sensitivity was 0.89 (95%CI: 0.79-0.95) and the pooled specificity was 0.88 (95%CI: 0.75-0.95).

Figure 4 displays the sROC curves of MA, showing the sensitivity of the individual articles mapped on the vertical scale, 1-specificity on the horizontal scale, with the summary (sensitivity, 1-specificity) point marked, as well as the summary ROC curve and the confidence region for the summary (sensitivity, 1-specificity) points. The area under the sROC curve was 0.7706 (SE: 0.0927) in non-benign cysts, 0.9248 (SE: 0.0691) in high-risk cysts, and 0.9555 (SE: 0.0293) in mucinous low-risk cysts. The results of the studies had greater variation in non-benign cysts as shown by the wide confidence region.

In the four studies, 566 patients had DNA available to perform MA in PCF. Pooled analysis (Figure 5) showed a diagnostic yield of 54.3% (95%CI: 49.8%-58.7%; I^2 = 39.605%; test for heterogeneity P = 0.174).



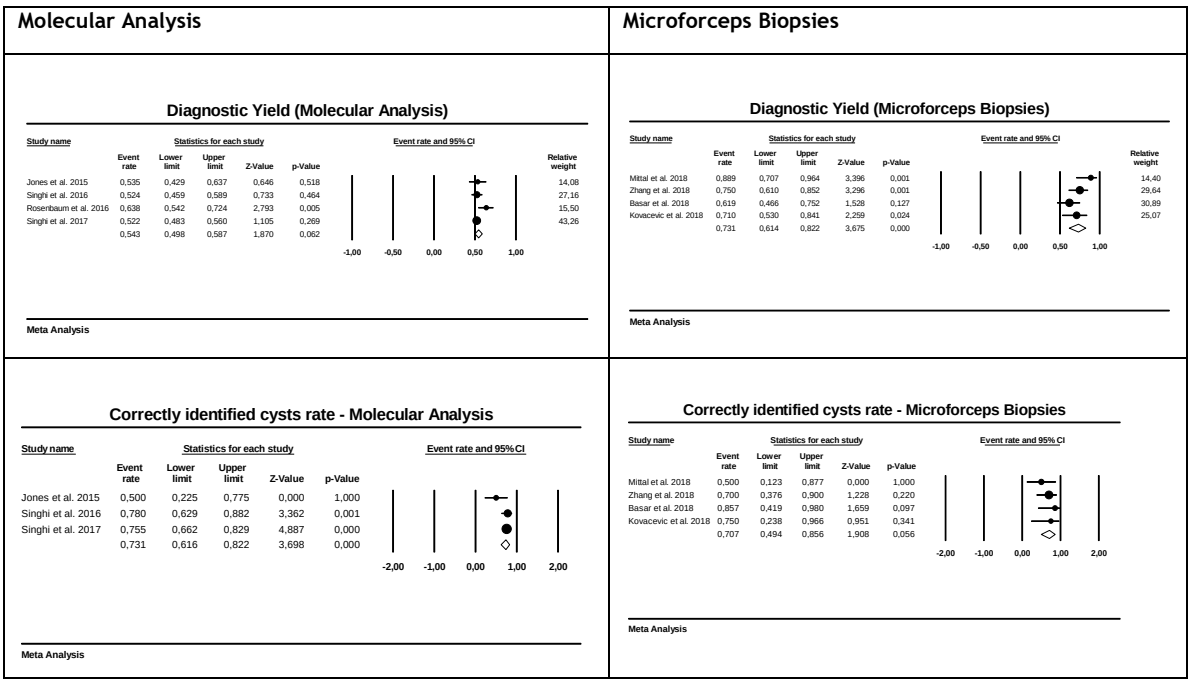


Figure 5. Forest plots of molecular analysis and microforceps biopsies on the secondary outcomes of this meta-analysis.

By considering the classification of cysts by specific type (IPMNs, MCNs, cNETs, SCAs, pseudocysts, ACCs, and other RCs), MA identified correctly 73.1% of cysts (95%CI: 61.6%-82.2%; I^2 = 37.381%; test for heterogeneity P = 0.203) (Figure 5).

Micro forceps biopsy: Four articles were included in the meta-analysis for diagnostic accuracy of histology obtained using MFB. Figure 6 shows the forest plots of sensitivity and specificity for the three subgroups of relevant cysts. The forest plots for MFB show variable specificities within the papers, from 0 to 1, which can be due to the small numbers of patients with the target condition in some studies.

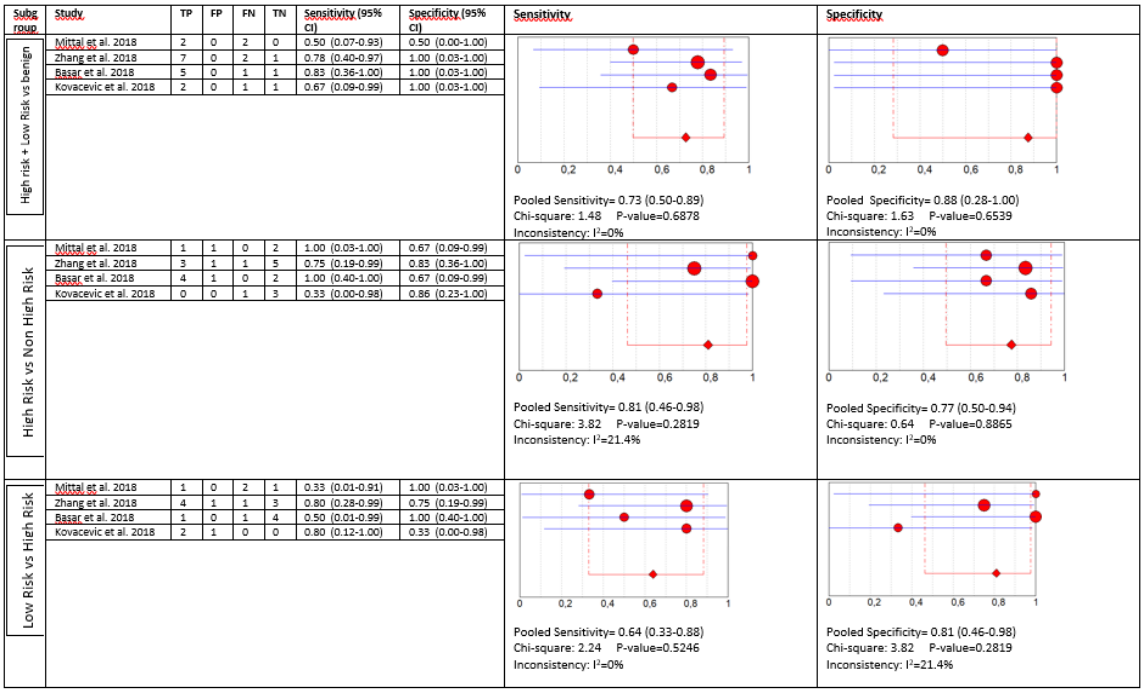


Figure 6 Forest plots of the included studies for microforceps biopsies. In parentheses are the 95% confidence intervals (CI) of the sensitivity and specificity. The figure shows the estimated sensitivity and specificity of the study (red circle) and its 95%CI (blue horizontal line). The area of the circle reflects the weight that the study contributes to the meta-analysis.

For each of the three subgroups there exists a low heterogeneity in sensitivity (I^2 = 0%, I^2 = 21.4%, I^2 = 0%) and specificity (I^2 = 0%, I^2 = 0%, I^2 = 21.4%), therefore fixed effect models were used. As presented in Figure 6, in the first subgroup the pooled sensitivity was 0.73 (95%CI: 0.50-0.89) and the pooled specificity was 0.88 (95%CI: 0.28-1.00). In the second subgroup sensitivity was 0.81 (95%CI: 0.46-0.98) with a specificity of 0.77 (95%CI: 0.50-0.94) and in the last subgroup the pooled sensitivity was 0.64 (95%CI: 0.33-0.88) and the pooled specificity was 0.81 (95%CI: 0.46-0.98).

The results were plotted as a symmetrical sROC curve (Figure 4). The area under the sROC curve was 0.7640 (SE: 0.1261) in the first subgroup, 0.8154 (SE: 0.098) in the second subgroup, and 0.7509 (SE: 0.1277) in the last subgroup.

By pooling the data of the four studies that investigated the use of MFB to obtain a histopathologic diagnosis, we obtained a diagnostic yield of 73.1% (95%CI: 61.4%-82.2%; I^2 = 47.774%; test for heterogeneity P = 0.125) (Figure 5).

By considering the outcome "specific cyst type" diagnosis, MFB correctly identified 70.7% of the cysts (95%CI: 49.4%-85.6%; I^2 = 0%; test for heterogeneity P = 0.056) (Figure 5).

DISCUSSION

In this meta-analysis we analyzed two different but promising tests to diagnose PCNs – molecular analysis and microforceps biopsy. To our knowledge this is the first study of this nature, and it included 1206 patients with PCNs of which 1058 underwent MA and 148 MFB. All patients had the index tests performed in PCF obtained pre-operatively, exclusively with NGS for MA and the Moray micro forceps biopsy device (US Endoscopy, Mentor, Ohio) used for MFB. We analyzed 203 cysts, 178 evaluated with MA and 25 with MFB, all referred for surgery, and with a surgical pathology specimen used as reference standard for diagnosis.

In this comparative analysis we included all studies, without restriction to simultaneous evaluation of both tests, because only one of such studies has been published[20]. This study, which includes 48 patients but only 10 surgical pathology specimens, showed identical results for MA and MFB in low-risk and high-risk cyst diagnosis, but higher specific cyst type diagnosis for MFB.

The data from the seven studies included in the meta-analysis, although with limited number of patients, particularly for MFB, suggests that MA is more accurate than MFB for diagnosis of PCNs, including high-risk and low-risk lesions. MA has superior accuracy to discriminate high-risk cysts from other PCNs and low-risk from high-risk neoplastic cysts. MA performance was considered excellent with AUC values of 0.92 and of 0.96 for high-risk and low-risk neoplastic lesions, respectively, as compared to MFB, which showed a fair or good performance, with an AUC of 0.81 and 0.75, respectively for the same lesions (Figure 4). The specificity of MA is good (0.88) but it has a low sensitivity (only 0.57) for high-risk cysts. This may be explained by technical issues, by low prevalence of relevant genetic mutations in malignant PCNs, or by mutations not included in the current NGS panels. The sensitivity and specificity are high (0.89 and 0.88, respectively) for MA when comparing low-risk to high-risk cysts, which reflects the genetic nature of pancreatic carcinogenesis with cumulative mutations from benign to malignant cysts[60].

For discriminating benign cysts from both low-risk and high-risk cysts, the performance of MA and MFB was identical and fair according to AUC values of 0.77 and 0.76, respectively. This non-superiority of MA in the diagnosis of benign cysts in this meta-analysis may be due to technique-inherent issues and/or under-representation of benign cysts in surgical series. In fact, “no genetic mutation” is considered a false negative result in most benign rare cysts, but some of these lesions (retention cysts, etc.) have no diagnostic genetic mutations. On the contrary, the most frequent benign cysts, SCAs, harbor a VHL mutation, exclusively present in these benign lesions and allowing for discarding a malignant lesion. In the MA studies, one third of rare benign cysts were classified as false negative results, due to absence of characteristic mutations (Table 1).

Another example of PCN that is not amenable to a MA diagnosis with current genetic panels is cNET, also reducing the accuracy of MA for diagnosis of high-risk cysts. The sensitivities were identical for MA and MFB (0.75 and 0.72), but the latter had higher specificity (0.73 and 0.88, respectively). Limited tissue sampling with MFB can explain the reduced sensitivity with robust specificity. As MA depends on denuded DNA in suspension in PCF, no sampling error is expected, which may explain its greater accuracy in neoplastic cysts, comparing to MFB.

Concerning secondary outcomes, even with the limitations of tissue sampling inherent to MFB, this meta-analysis showed that the diagnostic yield of MFB was superior to MA with rates of correctly identified cyst identical with MA and MFB (Tables 1 and 2). In fact, the definition of diagnostic yield, which for MA was “detection of genetic mutations”, may have led to a falsely low value due to the

presence of some rare types of benign cysts (retention cysts, lymphoepithelial cysts, epidermoid cysts, squamous cysts in two studies[30,113]) that have no characteristic diagnostic genetic mutations.

In clinical practice, patient symptoms, cyst imaging features, CEA, and cytology of PCF are required for diagnosis and decision for either treatment or surveillance according to cyst types[49]. PCF analysis, including CEA to distinguish mucinous from non-mucinous cysts and cytology to select those that harbor HGA or early pancreatic carcinoma and require surgical treatment, have suboptimal accuracies[84], due to scant cellularity and limited PCF volume. In this context, additional diagnostic tests are necessary to improve cyst classification and refine clinical decision. DNA markers require limited amounts of PCF, increasing the diagnostic yield[32,45,50,51], but with considerable technical complexity and costs. In fact, in routine clinical practice a major pitfall for PCNs diagnosis is the limited volume of PCF obtained, precluding routine pre-operative testing. As DNA analysis requires less volume of PCF, it may become an alternative test in these circumstances. This major advantage of molecular analysis was not possible to evaluate in this meta-analysis, because the volume of cystic fluid obtained in pancreatic cysts was not available in most studies analyzed.

As MA continues to evolve, questions remain about its accuracy, how it influences patient management, and in what order the analysis should be performed to better support clinical decisions. Previous studies[49] have shown that DNA testing combined with clinical features increased PCNs diagnosis compared to either alone. With multiple recent advances in biomarkers, molecular genetics will probably prove to be useful in the management of PCNs[138]. In a previous meta-analysis, pre-operative cytology of PCNs has shown low sensitivity for diagnosis[139], endorsing additional tests to improve diagnosis. Another meta-analysis of diagnostic accuracy of EUS-FNA with CEA and cytology analysis in differentiating mucinous cysts has demonstrated to be accurate to confirm the diagnosis but performed poorly in excluding it[13]. The role of *KRAS* as individual screening test has been analyzed before[55] with poor accuracy and added benefit coming from a combined approach with cytology. A recently published meta-analysis supporting *KRAS, GNAS,* and *RNF43* mutations as diagnostic markers of IPMNs[56] used different methods for mutation detection, different tumor materials, and clinicopathologic data as reference standard for diagnosis, which may limit its clinical application in evaluation of PCNs with mutational analysis performed only in PCF.

In this scenario, new markers are needed for PCNs stratification, and in our meta-analysis both MA and MFB have acceptable diagnostic accuracies. The two largest studies of MA[30,113] showed higher accuracy for diagnosis, which underscores the role of technical aspects of PCF collection, storage, and laboratory analysis for improved accuracy with this technique.

On the other hand, MFB provides tissue fragments for routine histological evaluation, without additional PCF required other than for standard analysis. The technical feasibility of through-the-needle microforceps biopsies revealed to be excellent, even in cysts located in the pancreatic head, despite the required 19-gauge caliber of the EUS-FNA needle. Another potential advantage of MFB is to allow the diagnosis of histologic subtypes of IPMNs, which can potentially be used for risk stratification[57], but still requires further validation.

Strengths and limitations

We applied strict exclusion criteria, with all analyzed patients having a surgical pathology specimen as the reference standard for diagnosis, because histopathology is the gold standard for diagnosis of neoplasia. Another major strength of this meta-analysis is having identical lesions (size and location) analyzed in both groups. These important strengths provide a more realistic accuracy estimate of the

tests evaluated. In previous studies of cytology including both surgical pathology and clinical follow-up[54] as reference standard, pooled sensitivities were 12% higher than in studies with exclusive surgical pathology[55] as reference standard in the diagnosis of mucinous cysts, with test accuracy overestimation. Finally, the pooled results have low heterogeneity.

The quality of a systematic review depends on the quality of studies included, and our quality assessment of patient selection regarding the risk of bias and applicability was high. As sensitivity and specificity are sensitive to study design and influenced by the spectrum of disease, sample collection, and processing, there may be a risk of bias and the results, although correct, their interpretation may be inaccurate. Moreover, there was incomplete reporting in one primary study, having no separate information on specific cyst type, mucinous or malignant cyst diagnosis[91], and the study was excluded from quantitative analysis. Although one study was excluded from the meta-analysis, MA with three studies included more patients (953, of whom only 153 in the surgical cohort) than the group of MFB with four studies but fewer patients (148, with only 25 in the surgical cohort). This can represent a surgical selection bias for both tests studied. Moreover, MFB studies were all retrospective, with small sample size, without pathology diagnosis for most benign and pre-malignant cysts, and non-consecutive patients that were selected on endoscopist discretion, which may have led to bias. Another limitation is the time between the index tests and the reference standard, because the final diagnosis could have been made at different time intervals from the tests. If the time between index tests and reference standard is too long, the true disease status of the patient may have changed by the time the reference standard was assessed. Aditionally, the different number of malignant cysts per study, particularly in the MA group, may have led to part of the heterogeneity in sensitivity and specificity. Finally, as MA does not increase the risks of standard EUS-FNA (the analysis is performed in remnant cystic fluid after standard diagnosis) we did not perform a safety analysis of MFB, but the four studies analyzed described only rare non-severe adverse events.

Future perspectives

With the increasing diagnosis of asymptomatic PCNs, most with potential for malignancy, there is a growing need to find accurate and affordable tests for diagnosis. The goal of management of patients with pancreatic cysts is to detect and resect cysts before progression of malignancy, while avoiding unnecessary follow-up procedures in benign cysts and surgery in low-risk PCNs.

Biomarkers of malignancy are promising, but clinicians should be aware of their current diagnostic performance limitations and type of lesions identified. In addition to significant costs, logistic difficulties in preserving material for future molecular analysis in busy general hospitals, and the technical complexity of the test, the generalized use of MA seems difficult in clinical practice. On the other hand, if MFB proves in larger studies to be safe and to allow tissue acquisition and gives the histological criteria needed for a correct diagnosis of PCNs, it may be immediately implemented in clinics, because the endoscopic procedure is standard, and histology is already a widespread procedure in clinics. MFB may be especially useful for benign lesions, for which both surgery and surveillance are unnecessary, representing a considerable burden in pancreas clinics due to current diagnostic limitations[58].

For MA to become relevant in routine clinical care in the future, its role in early cancer diagnosis and its prognostic value in PCNs requiring periodic surveillance must be confirmed. Also, for successful massive implementation, it is required to develop as an universal, highly accurate, first line test with clinical impact in cyst diagnosis, prognosis, and patient management. MA, both in PCF and peripheral blood, for standard analysis of multiple simultaneous biomarkers, allowing non-invasive diagnosis and

risk stratification of these lesions[59] would be valuable. For the present time, MA and MFB can only be recommended as complementary or as second line tests in case CEA and cytology of PCF are non-diagnostic. For both tests, large multicenter validation studies are still missing.

CONCLUSION

Our study confirms the diagnostic value of both MA and MFB, with higher diagnostic accuracy of MA than MFB for both low-risk and high-risk mucinous cysts. Genetic analysis should not be replaced by MFB in this context. Clinicians should be aware of the higher accuracy of MA for the diagnosis of malignant and high-risk cysts.

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publication bias, heterogeneity, and study quality.

2. Biomarkers for Diagnosis of Mucinous Pancreatic Cysts

2.1. Clinical impact of *KRAS* and *GNAS* analysis added to CEA and cytology in pancreatic cystic fluid obtained by EUS-FNA. Published in DDS.

Abstract

Background: Pancreatic cysts are common incidental findings, raising diagnostic and treatment dilemmas.

Aims: To determine the added value of *KRAS* and *GNAS* mutation analysis for cyst classification and decision making.

Methods: We analyzed 52 frozen samples of pancreatic cystic fluid (PCF) obtained by EUS-FNA between 2008-14. In addition to EUS with FNA cytology, CEA was determined and mutations of *GNAS* (exons 8 and 9) and *KRAS* (exons 2 and 3) genes were analyzed using Sanger sequencing

Results: 35/52 patients were females with a mean age of 59±15 years (29-91). Cysts were classified as mucinous in 21 (40%) patients (14 low-risk, 7 malignant) and non-mucinous in 31 (60%). After EUS-FNA, 11 patients were operated, 6 had chemotherapy or palliation, 1 had endoscopic drainage and 34 are on follow-up after a mean of 57 months. *KRAS* mutation was detected in 9 (17%) and *GNAS* in 2 (4%) samples. Patients harboring cysts with *KRAS* mutations were older (p=0.01), cysts were more commonly mucinous (p=0.001) and with a malignant cytology (p=0.01). *KRAS* mutations were present in both low-risk and malignant mucinous lesions. For identifying mucinous lesions, CEA >192ng/mL performed better (AUC ROC=93%), whereas for malignant/high-risk mucinous lesions, EUS imaging had the best accuracy (AUC ROC=88%). After molecular testing, re-allocation in cyst classification occurred in 10 patients, but correctly in 2 patients only.

Conclusions: In this cohort of patients with pancreatic cysts followed prospectively, *KRAS* and GNAS mutations had no diagnostic benefit in comparison with conventional tests.

Keywords

EUS-FNA; pancreatic cystic neoplasm; genetic analysis; CEA; DNA; KRAS or GNAS

Text

INTRODUCTION

Pancreatic cancer stands as the 4th leading cause of cancer death in the USA and it is expected to be the 2nd by 2030. Mucinous cysts are believed to be premalignant and would represent an excellent opportunity for early diagnosis in this malignancy [1]. However, the prevalence of pancreatic cysts over 1 cm in the general population is around 2% and cyst prevalence increases with age[2] making differential diagnosis of these lesions, a true challenge.

Pancreatic cysts include a wide range of diagnosis and can be non-neoplastic (pseudocysts) or neoplastic, which are the most frequent in clinical practice. According to WHO classification of pancreatic tumors, neoplastic cysts are classified as benign (serous cystadenomas - SCAs), pre-malignant (intra-papillary mucinous neoplasms – IPMNs and mucinous cystic neoplasms – MCNs, with low grade epithelial atypia - LG or high grade epithelial atypia - HG) and malignant lesions (ductal adenocarcinomas - ADCs, MCNs and IPMNs with an associated invasive carcinoma - IC, cystic neuroendocrine tumors – NETs, etc.).

In clinical practice, pancreatic cyst management starts with a distinction between non-mucinous from mucinous followed by low-risk versus high-risk/malignant mucinous lesions definition. The neoplastic benign/inflammatory cysts (SCAs, pseudocysts) do not progress to malignancy and require, at the most, conservative follow-up. Low-risk mucinous cysts (IPMNs and MCNS with LG) require surveillance due to malignancy risk. In high-risk mucinous/malignant lesions, including pre-malignant cysts (IPMNs and MCNs - HG) and malignant cysts (ADCs, IPMNs and MCNs - IC) surgery is indicated. We should be aware that rarely, non-mucinous cysts can be malignant (NETs) and require surgery. Cross-sectional imaging is not accurate in distinguishing different cyst types [3] and cystic fluid analysis with CEA determination and cytology are recommended despite limitations on pancreatic cyst discrimination [4,5,6,7].

Molecular analysis in PCF, particularly *KRAS* and *GNAS* mutational analysis showed promising results in mucinous cysts diagnosis[8]. Some studies show discrepant results [9,10] and the molecular makers impact in clinical practice remains unclear[11].

The aim of this study was to evaluate *KRAS* and *GNAS* mutational status in pancreatic cysts in a cohort of prospectively followed patients, to determine its accuracy in identifying mucinous cysts, particularly high-risk/malignant lesions, and to evaluate its added value in decision making.

METHODS

Cases were selected from the registry of EUS-FNA and PCF database of the Portuguese Institute of Oncology in Lisbon started in 2008, which is used for diagnosis and clinical management of patients with pancreatic cysts in our institution. All patients give informed consent for EUS-FNA and PCF analysis and storage. The present study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1143). After undergoing EUS-FNA, patients are evaluated in pancreas clinic, and referred for surgery (surgical cohort) or imaging follow-up, palliation or endoscopic drainage (non-surgical cohort). To evaluate *KRAS* and *GNAS* mutation distribution and the performance of mutation analysis in cyst diagnosis, three groups of lesions were defined according to surgical pathology or EUS-FNA cytology and prolonged clinical follow-up: Group 1) Non-mucinous cysts (NMC), including neoplastic benign/inflammatory cysts (SCAs, lymphangiomas and pseudocysts) and NETs; Group 2) Low-risk mucinous cysts (LRMC), including IPMNs and MCNs with LG; Group 3) High-risk/malignant mucinous cysts (HRMC), including ADCs, IPMNs and MCNs with HG. To understand its effect in decision-making, a cyst classification according to mutational status information was compared with the diagnosis based on CEA and cytology.

EUS-FNA PCF collection and storage

In all patients undergoing EUS-FNA for evaluation of a pancreatic cyst, the PCF obtained is immediately separated in two samples. Sample A (0.5 mL) is centrifuged for cytospin preparation for cytological analysis, and the supernatant fluid is sent for CEA and amylase evaluation; sample B (with the remaining volume of PCF) is immediately put on ice and stored at -80˚C in 0.25 mL aliquots, no more than 30 minutes after collection. A maximum volume of 0.25 mL was used for this molecular analysis.

Case selection

From 266 patients undergoing EUS-FNA for pancreatic cyst evaluation between 2008 and 2014, 102 frozen PCF samples were obtained and stored at -80˚C. For this study we performed molecular analysis in samples of 52 patients who had more than 1 mL (4 aliquots) of PCF stored. Clinical data, including cyst characteristics and treatment decision have been prospectively collected and registered.

EUS imaging

EUS images were reviewed and cystic morphology (thick septs, mural nodules, wall thickening or mass) and main pancreatic duct features (dilatation >10mm or cyst communication) documented. According to imaging, cysts were broadly classified in three groups: 1) NMC (cases suggesting SCAs or with no septs or nodules and features of pancreatitis); 2) LRMC (< 3cm, no wall thickening, mural nodule or mass); 3) HRMC (>3 cm, wall or sept thickening, mass, mural nodule or dilatation of Wirsung >10mm).

PCF analysis of CEA and cytology

In all 52 patients, 0.5 mL of PCF was submitted for CEA determination. A level greater than 192 ng/mL prompted a classification of a mucinous cyst and lower than 192 ng/mL of a non-mucinous cyst. Cytological analysis of PCF classified the cysts in one of the three previously defined groups. The presence of atypical cells in cytology defined a cyst as HRMC. In patients with a non-diagnostic cytology, cyst classification was based on CEA, imaging and long-term follow-up.

Treatment decision

The treatment decision was accordantly with the consensus guidelines of Sendai 2006[12] revised in Fukuoka in 2012[13]. High-risk cysts due to suspicious imaging (size >30 mm and/or mass/mural nodule/thick wall) or a malignant cytology (invasive cancer, NETs), HG epithelial atypia or suspicious cytology (atypical cells) in good surgical candidates, were referred for surgery. LRMC or NMC were kept on follow-up. To patients with invasive or locally advanced lesions and a positive/malignant cytology were offered palliation. One patient with a symptomatic pseudocyst had endoscopic drainage.

***KRAS* and *GNAS* mutation analysis**

DNA was isolated from 250 µl of PCF using the Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen Biotek Corp., ON, Canada). Mutational analysis of *KRAS* (exons 2 and 3) and *GNAS* (exons 8 and 9) was performed by Sanger sequencing. The exons were PCR amplified in a standard PCR buffer (Invitrogen, Waltham, MA, USA) using specific primers. Sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the respective products analyzed on the ABI PRISM™ 3130 Genetic Analyzer, using the Sequencing Analysis software v3.4.1 (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

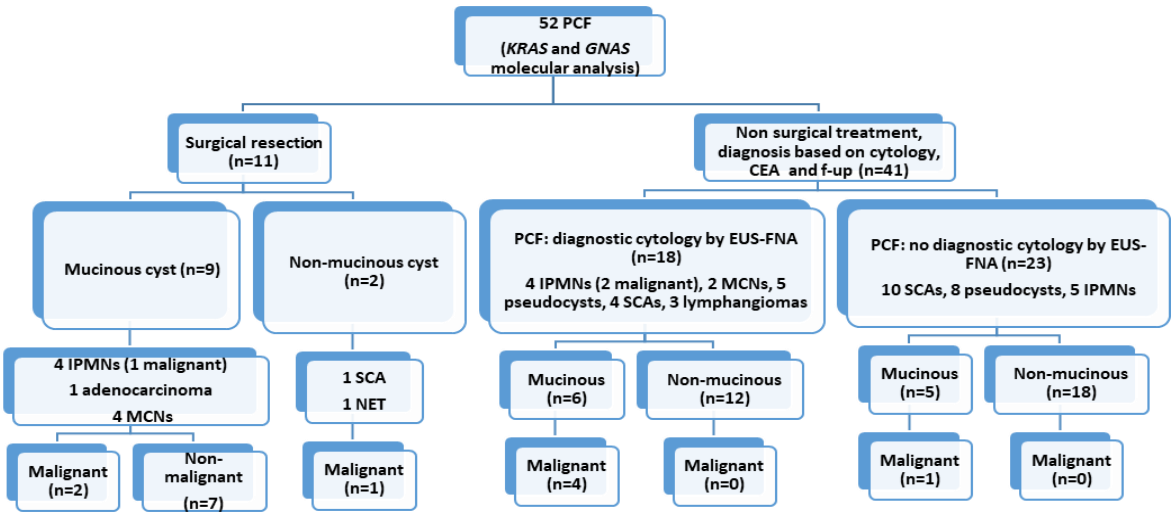
Descriptive data are expressed as mean ± SD. Fisher's exact test was used to assess differences between DNA-mutant and DNA-wild type cysts for dichotomous variables. All tests were two-sided and statistical significance was defined as a p-value < 0.05.

The sensitivity, specificity, positive and negative predictive value and diagnostic accuracy of EUS imaging, CEA level, cytology and *KRAS* and *GNAS* mutational status in PCF were evaluated for the diagnosis of mucinous cysts and high-risk mucinous/malignant cysts. Receiver operator curves were generated and area under the curve (AUC) was calculated. Pancreatic cyst final diagnoses were based on surgical specimens or, in the non-surgical cohort, on PCF CEA, cytology and outcome combination after prolonged follow-up. Statistical analysis was performed using SPSS Statistics version 23.

RESULTS

Demographics, cystic lesion standard analysis and clinical decision

Between 2008-14, 52 patients who underwent EUS-FNA for evaluation of a pancreatic cyst and who had at least 1 mL of PCF stored at -80º, had one aliquot of 0.25 mL retrieved for molecular analysis. The study population characteristics are displayed on Table 1 and the flow chart with clinical management is detailed on Figure 1.



KRAS, oncogene of Kirsten rat sarcoma virus; *GNAS*, gene of guanine nucleotide binding protein, alpha stimulating; IPMNs, intrapapillary mucinous neoplasms; MCN, mucinous cystic neoplasms; SCAs, serous cystadenomas; PCF, pancreatic cyst fluid; Low-risk lesion, means cytology with low grade or intermediate grade dysplasia; Malignant, stands for high grade dysplasia, invasive carcinoma or NET;

Figure 1- Flowchart showing treatment decision after EUS-FNA. Surgical pathology or EUS-FNA (CEA ± cytology) and prolonged follow-up was used for final cyst classification as mucinous *vs* non-mucinous and malignant *vs* non-malignant (low-risk).

Table 1. Demographics and clinical characteristics of the study population.

Female gender, n (%) (n=52)	**35 (67,3%)**
Mean age at EUS-FNA, y, mean ± SD (interval)	**59,1 ± 14,8 (29-91)**
Cyst location, n (%) (n=52)	
Head	**22 (42,3%)**
Body	**20 (38,5%)**
Tail	**9 (17,3%)**
Multiple cyst locations	**1 (1,9%)**
Cyst size, cm, mean ± SD (interval)	**3,9 ± 2,3 (1-10)**
Cyst size >3 cm, n (%)	**29 (55,8%)**
Cyst with nodule/mass, n (%)	**18 (34,6%)**
EUS Imaging, n (%) (n=52)[14]	
No high risk features	**13 (25%)**
1 high risk feature	**29 (55,8%)**
≥2 risk features	**10 (19,2%)**
PCF CEA, n (%) (n= 52)	
CEA <192 ng/mL, n (%)	**31 (59,6%)**
CEA ≥192 ng/mL, n (%)	**17 (32,7%)**
No result available	**4 (7,7%)**
PCF cytology, n (%) (n=52)	
Non-diagnostic	**27 (51,9%)**
Negative for malignancy	**14 (26,9%)**
Suspicious/malignant	**10 (19,2%)**
NET	**1 (2%)**
Treatment decision, n (%) (=52)	
Follow up	**34 (65,4%)**
Surgery	**11 (21,2%)**
Endoscopic drainage	**1 (1,9%)**
Palliation (symptomatic or chemotherapy)	**6 (11,5%)**

EUS-FNA, Endoscopic ultrasound with fine needle aspiration; High-risk features: cyst size ≥3 and solid component or thick wall or dilated Wirsung (>10mm); CEA, carcinoembryonic antigen; NET, neuroendocrine tumor; PCF, pancreatic cyst fluid; SD, standard deviation

In our series, 35/52 patients were females, with a mean age of 59 years old, with cysts located predominantly in pancreatic head and body, and a mean size of 3.9 cm. Most were incidental findings, with 75% (39/52) of asymptomatic patients, 23% (12/52) with complaints of abdominal pain/dyspepsia and 2% (1/52) with vomiting. No patients presented with pancreatitis or jaundice.

After EUS-FNA, PCF was sent for CEA and cytology in all cases. A CEA determination was obtained in 92% (48/52) and a conclusive cytology in 48% (25/52) of PCFs.

After clinical, imaging and standard cystic fluid analysis, 31/52 (60%) lesions were classified as non-mucinous and 21/52 (40%) as mucinous. On the whole series, 8 (15%) cysts were malignant, with 7 mucinous malignant cysts and 1 NET. Clinical decision, after EUS-FNA, was surgical resection in 11 (21%) patients, palliation in 6 (11,5%), endoscopic drainage in 1 (2%) and 34 with low-risk cysts were referred for follow-up. In the 11 operated cysts, there were 3 malignant cysts (1 ADC, 1 IPMN-IC, 1 NET), 7 mucinous low-risk (3 IPMN-LG, 4 MCN-LG) and 1 benign cyst (1 SCA).

The mean follow-up time in this series is 45±36 (3-40) months and even longer in 34 patients on follow-up with a mean of 57±35 (14-156) months. Of these 34 patients, 5 (10%) with benign/inflammatory cysts stopped follow-up (SCNs, lymphangiomas) or were lost for follow-up and 29 (56%), are still on follow-up with no morphological changes in cystic lesions. In this series, 5 (9.6%) patients died (3 of pancreatic cancer, 1 from another neoplasia and one as complication of pancreatic surgery).

Molecular analysis

KRAS and *GNAS* mutational analysis was performed in the 52 patients after clinical decision and did not interfere with it in any instance. In 5/52 samples no DNA amplification occurred in at least one of the exons analyzed and the test was considered non-informative. There were only two samples with a *GNAS* mutation and in both a *KRAS* mutation was also present, one in an IPMN-LG and the other in an unressectable adenocarcinoma referred for chemotherapy.

Table 2 presents clinical, imagiological and PCF analysis, clinical decision and definitive diagnosis in 9 cysts with *KRAS*/*GNAS* mutations.

Table 2. Detailed clinical, imagiological, biochemical, cytologic features and final diagnosis of 9 mutated *KRAS*/*GNAS* cysts.

Patient	Gender	Age	Clinical Symptoms	Cyst size (cm)	Cyst location	Ductal dilation	Mural nodule or mass	CEA (ng/mL)	Malignat cytopathology	KRAS mutation	GNAS mutation	Clinical Decision	Diagnostic Surgical Pathology	Diagnostic Cytology EUS-FNA
1	Female	81	Asymptomatic	1,9	Body	No	Absent	300 754	Neoplastic: Benign	Exon 2 G12A	Wild type	Follow-up (84 Months)	No (EUS-FNA and F-up)	IPMN LG
2	Female	64	Pain	1	Body	No	Present	11 764	Positive	Exon 2 G12D	Wild type	Chemotherapy	No (EUS-FNA and F-up)	AdenoCa
3	Female	63	Pain	6	Body	No	Present	3802	Positive	Exon 2 G12D	Exon 8 R201H	Chemotherapy	No (EUS-FNA and F-up)	AdenoCa
4	Male	75	Pain	2	Head	No	Absent	10 003	Positive	Exon 2 G12D	Wild type	Surgery	AdenoCA in IPMN	AdenoCa
5	Male	62	Asymptomatic	2,1	Tail	No	Absent	125	Non-diagnostic	Exon 2 G12D	Wild type	follow-up (48 Months)	No (EUS-FNA and F-up)	Pseudocyst/ Probable IPMN
6	Male	65	Asymptomatic	3	Body	No	Absent	249	Non-diagnostic	Exon 2 G12R	Exon 8 R201H	Surgery	IPMN-LG	
7	Male	80	Pain	2,8	Tail	No	Present	150 490	Positive	Exon 2 G12V	Wild type	Chemotherapy	No (EUS-FNA and F-up)	AdenoCa
8	Female	78	Pain	5	Head	Yes	Present	191 437	LG	Exon 3 E61H	Wild type	Paliation	No (EUS-FNA and F-up)	AdenoCa
9	Female	60	Asymptomatic	3,7	Tail	No	Present	1 515	Atypical cells	Exon 3 E61H	Wild type	Surgery	MCN LGD	Atypical cels

Although 5/9 patients with *KRAS* mutated cysts had an invasive adenocarcinoma, the remaining 4 patients had low-risk mucinous cysts. All 5 patients with malignancy were symptomatic, PCF had CEA >192 ng/ml, cytology was positive for malignancy in 4/5 patients and EUS high-risk features were present in 4/5 patients as well.

Table 3 summarizes patients' and cysts' characteristics in the 47 patients with informative KRAS mutational analysis.

Table 3. Clinics and characteristics of *KRAS*-wild type and *KRAS*-mutant cystic lesions.

Patient and cyst characteristics (n=47*)	***KRAS*-wild type**	***KRAS* mutant**	***p*-value**
Gender, n			
Female	27	5	0,438
Male	11	4	
Age (y)			
Mean ± SD	56,5±14,6	69,2±7,8	0,016
Cyst Size (cm)			
Mean± SD	4,1±2,3	2,9±1,7	0,171
Cyst Location, n			
Head	17	3	
Body	14	3	
Tail	6	3	
Multiple locations	1	0	0,647
EUS – Diagnosis			
Non-mucinous	25	4	
Low-risk mucinous	12	3	
High-risk mucinous	1	2	0,085
CEA (n= 43)			
CEA <192 ng/mL	28	1	
CEA >192 ng/mL	7	7	0,001
EUS-FNA cytological diagnosis			
Non-diagnostic	22	3	
Negative for malignancy (inflammatory)	5	0	
Atypical	3	1	
Neoplastic: Benign or Other	5	1	
Positive/malignant cells	2	4	0,011
Surgical Specimens (n=10)			
MCN - LG	3	1	
IPMN- LG	1	1	
Adenocarcinoma/IPMN - IC	1	1	
NET	1	0	
SCA	1	0	0,374

SD, standard deviation; IC, invasive carcinoma; LG, low grade; *Only 47 PCF with conclusive mutational analysis in all the 4 exons of *KRAS* and *GNAS* studied were considered for analysis.

There was no gender preponderance in patients harboring cysts with *KRAS* mutations, but the latter were significantly older (69.2 vs 56.5 years, p=0.01). No significant differences were found in cyst size or location, but mutations were more frequent in mucinous cysts (CEA >192 ng/mL, p=0.001). In lesions with EUS high-risk features, no significant differences were detected (p=0.08). In the 10 patients of this subgroup that were submitted to surgery (2 NMC, 6 LRMC and 2HRMC), no association was found between *KRAS* mutation and surgical pathology diagnosis.

To understand the discriminant power of molecular analysis between the three categories of cystic lesions previously described, we analyzed the 29 patients with a definitive pathologic diagnosis, either with a surgical pathology specimen or EUS-FNA cytology and a prolonged follow-up. Table 4 shows that

KRAS/GNAS mutations were more frequent in mucinous lesions, both LRMC and HRMC, as compared with NMC, which were all *KRAS* WT (p=0.045).

Table 4. Distributions of *KRAS* mutation in the three groups of cystic lesions (were considered only cysts with a EUS-FNA cytology and prolonged follow-up or final pathology diagnosis).

N=29	Mutant KRAS/GNAS	Wild type	Non-Diagnostic
Non-mucinous cysts (SCA, pseudocyst, NET, lymphangioma) (n=12)	0	11	1
Mucinous - LR Cysts (n=10)	3	6	1
Mucinous – HR/malignant cysts (n=7)	5	2	0

Table 5 shows the performance of the several tests, alone or in combination, to diagnose LRMC.

Table 5. Performance characteristics of EUS imaging, cystic fluid CEA, cytology and molecular analysis for mucinous cysts identification.

Mucinous cysts (10 LRMC+7 HRMC)/29	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95%CI)	Accuracy (95% CI)	Area under the ROC (CI)
EUS – Imaging	53 (28-77)	83 (52-98)	82 (54-95)	56 (42-67)	66 (44-82)	0,68 (0,48-0,88)
CEA > 192 ng/mL	93 (66-100)	92 (62-100)	93 (66-99)	92 (63-99)	92 (75-99)	0,93 (0,83-1)
Cytology	56 (28-77)	83 (52-98)	82 (54-95)	56 (42-69)	66 (46-82)	0,54 (0,31-0,77)
Mutational Analysis (*KRAS/GNAS*)	50 (25-75)	100 (72-100)	100	58 (46-69)	70 (50-86)	0,72 (0,52-0,92)
CEA ↑ or cytology + or Mutational Analysis +	94 (67-100)	77 (46-95)	83 (65-93)	91 (54-99)	86 (68-96)	0,84 (0,68-1)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; ROC, receiver-operating characteristics

CEA has the highest sensitivity and specificity and should be the reference test (AUC=0.93). Molecular analysis has the second better performance (AUC=0.72), with a low sensitivity (S=50%) but a specificity of 100%. However, in clinical practice, the most relevant distinction is between low-risk and high-risk mucinous cysts, as only the latter should be operated. Results for high-risk mucinous cysts are shown on Table 6.

Table 6. Performance characteristics of EUS imaging, cystic fluid CEA, cytology and molecular analysis for mucinous HR/malignant pancreatic cysts identification.

HRMC= 7/29	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95%CI)	Accuracy (95% CI)	Area under the ROC (CI)
EUS – Imaging	88 (47-100)	81 (58-95)	64 (41-81)	94 (73-99)	83 (64-94)	0,86 (0,68-1)
CEA >192 ng/mL	86 (42-100)	58 (34-80)	43 (29-58)	92 (63-99)	65 (44-83)	0,65 (0,44-0,86)
Cytology	67 (30-93)	90 (68-99)	75 (43-92)	86 (70-94)	83 (64-94)	0,79 (0,57-1)
Mutational Analysis (*KRAS/GNAS*)	63 (25-91)	82 (60-97)	63 (34-84)	83 (68-93)	78 (58-91)	0,73 (0,48-0,93)
CEA↑ or cytology + or Mutational Analysis +	88 (47-100)	57 (34-78)	44 (31-58)	92 (65-99)	66 (46-82)	0,79 (0,62-0,95)
EUS-Imaging + or Cytology +	100 (63-100)	71(48-89)	57 (40-72)	100	79 (60-92)	0,86 (0,72-0,99)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; ROC, receiver-operating characteristics

EUS-imaging has the best diagnostic performance (AUC=0.86), and the combination of EUS-imaging and cytology have better performance than full cystic fluid analysis with CEA, cytology and mutational analysis associated.

Molecular testing information on clinical decision making considering the whole series

When evaluating the diagnostic advantage of molecular analysis over conventional methods in discriminating between high-risk, low-risk and non-mucinous cysts, we observed that the 9 *KRAS*/*GNAS* mutations occurred both in low-risk and high-risk mucinous cysts. These were diagnosed as such using conventional methods, except for one *KRAS* mutation observed in a patient with a presumed pseudocyst. Table 7 summarizes this information.

Table 7. Molecular analysis results in low-risk mucinous, high-risk mucinous and non-mucinous cysts as classified after clinical, imaging, and CEA and cytology obtained by EUS-FNA.

52 cysts	LRMC (n=14)	HRMC (n=7)	NMC (n=31)
KRAS wild type	9	2	27
KRAS mutation	3	5	1
KRAS non-diagnostic	2	0	3
Total	14	7	31

LRMC, low-risk mucinous cyst; HRMC, high-risk mucinous/malignant cyst; NMC, non-mucinous cyst

Analysis of molecular testing according to clinical decision in the 52 patients, shows that 11 underwent surgery (2 with *KRAS* and 1 with *KRAS*/*GNAS* mutation), 6 had palliation/chemotherapy (3 with *KRAS* and 1 with *KRAS*/*GNAS* mutation), 1 was submitted to endoscopic drainage and 34 referred for follow-up, with no change in cyst characteristics or size after a mean time of 57±35 (14-156) months, including 2 patients with *KRAS* mutations in low-risk mucinous cyst (84 months of follow-up) and in an inflammatory cyst (48 months of follow-up), both stable.

Table 8 shows the changes in cyst classification according to molecular diagnosis.

Table 8. Effect of Molecular Analysis in cyst re-classification after conventional classification using clinical, imaging, and PCF CEA and cytology obtained by EUS-FNA.

AFTER EUS-FNA (N=52)	KRAS non-diagnostic n/N(%)	Supported n/N (%)	Changed n/N %	Correct Change n/N (%)
LRMC (N=14)	2/14 (14,3%)	3/14 (21,4 %)	9/14 (64,2 %)*	0/9 (0%)
HRMC (N=7)	0/7 (0%)	4/7 (57,1 %)	3/7 (42,8 %)**	1/3 (33,3%)
NMC (N=31)	3/31 (9,7%)	27/31 (87,1%)	1/31 (3,2 %)***	1/1 (100%)

LRMC, low-risk mucinous cyst; HRMC, high-risk mucinous/malignant cyst; NMC, non-mucinous cyst
*3 MCNs and 1 IPMN operated and 5 cysts on f-up, with CEA ↑ and cytology with inflammatory cells (2) and non-diagnostic (2) and benign cells (1). **3 operated cysts, 1 ADC, 1 MCN-HGD, 1 SCA (with a preoperative false positive cytology).***1 pseudocyst (CEA=125, non-diagnostic cytology).

In the LMRC, 6/12 would be classified as "non-mucinous" according to *KRAS* status which would be false in all cases. In contrast, 3/8 HRMC would be classified as "non-malignant" but this would be correct in 1/3 lesions only. This was in fact a false positive cytological diagnosis of malignancy, in a 56 years old female with a 2.9 cm cyst, CEA in PCF lower than 0.5 ng/mL, who was referred for surgery, with a final surgical pathology diagnosis of a SCA. Also, one change would occur in a lesion classified as a pseudocyst which, after molecular testing, was correctly re-classified as a low-risk mucinous cyst. This cyst was diagnosed in a 62 years old male, has a stable size of 2.1 cm after 48 months of follow-up, and had a CEA=125 ng/mL with a non-diagnostic cytology. It would be classified as a pseudocyst with standard PCF analysis, but is probably an IPMN with the *KRAS* mutation detected, with added value in cyst classification. IPMNs can be under-diagnosed with the CEA cut-off of 192 ng /mL.

Globally, only 2/10 (20%) of changes in cysts re-allocation due to mutational analysis are valuable for better cyst classification, and allow increasing low-risk mucinous cysts diagnosis, previously classified as inflammatory and not confirming a high-risk cyst with a false positive malignant cytology.

DISCUSSION

In our prospective cohort of patients with pancreatic cysts followed over a mean period of almost 5 years and managed according to published guidelines, we observed that *KRAS* and *GNAS* mutations in PCF added very little when compared to standard clinical exams – EUS-FNA with cytology and CEA. Molecular testing was able to distinguish between mucinous and non-mucinous lesions (AUC=0.72), although values of CEA >192 ng/mL was the most accurate test to identify mucinous lesions (AUC=0.93). Regarding the most clinically significant, high-risk mucinous/malignant lesions, EUS imaging alone or combined with a diagnostic cytology were more accurate than molecular markers for diagnosis (AUC=0.86 vs 0.73).

Expanded use of computed tomography and magnetic resonance resulted in increased detection of pancreatic cysts, some of which are believed to be precursors of pancreatic adenocarcinoma. Mucinous cysts have the potential to progress to malignancy [6], and current guidelines recommend surgical resection in "high-risk cysts"[13]. IPMNs can progress from low, to intermediate, to high-grade dysplasia, and ultimately to invasive carcinoma [8]. It is recommended that only IPMNs with high-grade epithelial atypia or an associated invasive carcinoma should undergo resection, while IPMNs with low-grade epithelial atypia can undergo surveillance.

However, cross-sectional imaging is not accurate in distinguishing different cyst types[3] and cystic fluid analysis with CEA and cytology are imperfect for cyst evaluation[4,5,6,7]. In our series, CEA was measurable in 90% of samples but cytology was informative only in 48% of cases. The accuracy of cytology from EUS-FNA samples ranges from 54% to 97%, but may be lower in smaller cysts[6]. In a recently published meta-analysis, CEA was found to have a sensitivity of 63% and a specificity of 88% to identify mucinous cystic tumors[5]. As cystic fluid CEA and cytology are considered investigational in some of the current guidelines[13], in clinical practice, management of pancreatic cysts remains difficult and highly individualized.

The high frequency of pancreatic cyst detection, the significant efforts for cyst follow up and the significant morbidity and mortality of pancreatic surgery for a possible benign disease make an accurate pancreatic cyst classification critical in clinical practice.

Molecular analysis, particularly *KRAS* and *GNAS* mutational status in PCF has shown to be useful for cyst classification and advanced neoplasia detection [15,16,17,18,19], but its true value in decision making remains unclear. Some retrospective studies[15], although including large numbers of operated patients and a large panel of molecular markers, do not mimic a real life scenario, where the physician is faced with patients carrying a pancreatic cyst and in whom he has to decide whether to recommend surveillance or surgery using clinical, imagiological, cytological and biochemical information. Our study represents a real life practice scenario and patients were all managed according to Sendai [12] or Fukuoka [13] guidelines. In agreement with more recent and restrictive guidelines [20], few patients were operated (11/52) and the majority (34/52) are on follow-up for a mean period of almost 5 years. Of note, cystic fluid was obtained from EUS-FNA and not during operation as in some other series [15]. Most of these doubtful lesions are small and aspirated volume is usually scant. In contrast with studies including solely operated patients [15,16], it is important to examine what happens to patients with *KRAS*

positive lesions who are not operated. In the present series 2/9 patients harboring *KRAS* mutated lesions are alive and stable after 84 and 48 months of follow-up, respectively.

Our study is in agreement with previous publications [21], which showed that *KRAS* mutations were highly specific to identify mucinous lesions. *KRAS* mutation had 100% specificity, an acceptable discriminative power (AUC=0.72), but a low sensitivity (50%). A recently published large prospective study of DNA-molecular testing in PCF also prior to surgery, demonstrated the limitations of Sanger sequencing compared to Next Generation Sequencing (NGS) in pancreatic cyst evaluation [18], with a sensitivity and specificity for mucinous cysts diagnosis of 89% e 100% for NGS and 65% and 100% for Sanger sequencing. The lower prevalence of *KRAS* mutations found in the present study (17%) can be related with the cyst types in our series, low PCF volume and the use of Sanger sequencing that presents a lower sensitivity when compared with NGS. However, we should remember that increasing technique sensitivity may increase the number of false positives, possibly increasing unnecessary surgeries. In fact, whereas a pancreatic mucinous lesion was previously considered worrisome, we now know that most of these lesions will not progress to malignancy [22] and the critical issue for patient care is diagnosing high risk mucinous/malignant mucinous lesions. In our study, the most accurate tests to diagnose these lesions were EUS-imaging findings associated whenever possible to cytologic samples. The added benefit of molecular markers in the present series was marginal – only 2/10 patients had a correct re-classification of their cystic lesions after molecular analysis, that would not affect clinical decision in either.

In our study *GNAS* mutation did not improve diagnosis. In fact, only 2 samples had a *GNAS* mutation and in both patients a concomitant *KRAS* was present, which leads us to conclude that in contrast to previous series, *GNAS* mutations are not sensitive to diagnose IPMNs.

One additional question is that some malignant cysts, as NETs, are not mucinous. In these lesions CEA level is low, *KRAS* is not usually mutated, and cytology is fundamental for decision making. This further reinforces that a combined or sequential analysis strategy must be the rule in pancreatic cysts. It makes sense to use the most sensitive method of diagnosis in young and healthy patients and to obtain more specific markers in older patients with comorbidities. In fact, in our surgical cohort of 11 patients, 1 surgery could have been avoided (1 SCA) and 7 surgeries, in low-risk mucinous cysts, at least delayed.

This study has several limitations. First, only 11/52 patients were operated and have an available surgical specimen. However, in the additional 18 patients with EUS-FNA cytology specimens, the extended period of observation allows us to assume the low-risk nature of the lesions. Second, as the samples selected had a stored volume over 1 mL, there may be a selection bias for larger cysts, but these are also those that raise more management problems. Finally, we may have an under diagnosis of mucinous cysts due to the cut-off CEA level of 192 ng/mL. In our series, 32 cysts had a CEA<192 ng/mL with a mean CEA level of 26.8±33 ng/mL (0.5-125) of which 13 had CEA ≤5 ng/mL. We only had one *KRAS* mutation in these 32 cysts, a cyst with CEA=125 ng/mL and a non-diagnostic cytology, with one additional mucinous cyst diagnosis.

In summary, our results do not support *KRAS* and/or *GNAS* mutational analysis using Sanger sequencing in pancreatic cysts. Although we found that *KRAS* mutations occur predominantly in mucinous and malignant cysts, CEA level for low-risk mucinous cysts and combined imaging and cytology for high-risk mucinous/malignant cysts are more accurate and should be for now recommended in clinical practice. Sanger sequencing, due to its low sensitivity, should be replaced by NGS multigenic panels. Molecular analysis cost-efficacy must be evaluated in real-life scenarios and in PCF obtained by EUS-FNA prior to decision making.

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2.2. Excellent Accuracy of Glucose Level in Cystic Fluid for Diagnosis of Pancreatic Mucinous Cysts. Published in DDS.

Abstract

Background: CEA in pancreatic cystic fluid (PCF) is standard for mucinous cysts diagnosis. Glucose is an alternative, but its accuracy remains poorly described.

Aims: To evaluate PCF glucose using a glucometer and compare its accuracy with CEA for mucinous cysts diagnosis.

Material and Methods: In frozen PCF obtained by EUS-FNA glucose was evaluated using a glucometer. CEA and cytology were available as standard of care. The accuracy of glucose and CEA were calculated using receiver operator (ROC) curves. Definitive diagnoses were surgical or clinicopathological.

Results: We evaluated 82 patients with a mean age of 61.3±14.8 years (25-91), predominantly (59%) females. Diagnoses included 17 serous cystadenomas, 5 pseudocysts, 20 intraductal papillary mucinous neoplasms, 3 mucinous cystic neoplasms, 5 adenocarcinomas, 4 neuroendocrine tumors, 2 other types, 26 non-defined. The median glucose levels (interquartile range) were 19 mg/dL (19-19) in mucinous and 105 mg/dL (96-127) in non-mucinous cysts ($p<0.0001$). The median CEA level was 741 ng/mL (165-28567) in mucinous and 9 ng/mL (5-19) in non-mucinous cysts ($p<0.0001$). For mucinous cyst diagnosis, a CEA>192 ng/mL had a sensitivity of 72% (95%CI:51-88), a specificity of 96% (95%CI:82-100), and ROC analysis showed an area under the curve (AUC) of 0.842 (95%CI:0.726-0.959), while glucose<50 mg/dL had a sensitivity of 89% (95%CI:72-98), a specificity of 86% (95%CI:67-96), and an AUC of 0.86 (95%CI:0.748-0.973). Pseudocysts presented low glucose, identically to mucinous cysts, with CEA allowing differential diagnosis.

Conclusion: Glucose measured by a glucometer is accurate for mucinous cyst diagnosis, with significantly higher levels in non-mucinous cysts, except pseudocysts.

Keywords

Glucose, CEA, pancreatic cyst, EUS-FNA, IPMN, MCN.

Text

INTRODUCTION

The widespread use and technical advances of abdominal imaging allied with population ageing, has led to an increase in the detection of asymptomatic pancreatic cystic lesions (PCLs). [1] PCLs encompass a wide spectrum of diagnoses that range from benign/inflammatory lesions [e.g. serous cystadenomas (SCAs), pseudocysts] to pre-malignant [intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs)], and malignant cysts [cystic adenocarcinomas (ADCs), cystic neuroendocrine tumors (NETs), acinar cell carcinomas, etc.]. The differential diagnosis between PCLs is important, since the clinical approach differs, with surgery required for high-risk and malignant cysts, surveillance for premalignant lesions, and no follow-up recommended for benign cysts. Currently, the diagnosis of these lesions includes pancreatic cystic fluid (PCF) analysis obtained by endoscopic ultrasound with fine-needle aspiration (EUS-FNA). [2,3]

Pancreatic mucinous cysts are precursors of pancreatic cancer including MCNs and the most frequent IPMNs, and the differential from other PCLs is not trivial.[4] Their malignant potential in surgical series encompasses 10% of non-invasive and 13% of invasive carcinomas.[5]

The preoperative diagnosis of mucinous cysts is based on an elevated carcinoembryonic antigen (CEA) level in PCF, and several pitfalls have been reported with this biomarker. First, the cutoff levels vary between laboratories,[6,7,8] limiting its reproducibility and making the interpretation of CEA results difficult. Second, a significant volume of PCF [at least 200 microliters (μl)], is required for CEA analysis, precluding its measurement when PCF is scant. Finally, the currently used CEA level (>192 ng/mL) has limited sensitivity for mucinous cyst diagnosis, with considerable overlap in CEA levels between mucinous and non-mucinous cysts.[7] To overcome these issues and increase the diagnostic yield, additional biomarkers have been analyzed in PCF, but these increase the complexity and costs considerably.[9,10,11,12]

An exploratory metabolomics study revealed that low levels of glucose in PCF had diagnostic value for identifying mucinous cysts.[13] This finding was later confirmed in two clinical studies with laboratorial and/or glucometer measurement of glucose in PCF.[14,15] However, both studies used aspirates mostly from surgically resected lesions, a situation that differs from real life practice, in which we need a pre-operative diagnosis.

The goal of our study is to evaluate the accuracy of glucose level measured with a glucometer, in PCF collected preoperatively by EUS-FNA, for diagnosis of mucinous cysts, and to compare its accuracy with that of laboratorially obtained CEA level.

PATIENTS AND METHODS

Sample acquisition and case selection

This longitudinal cohort study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1225).

We selected PCF samples from our Endoscopic Ultrasound registry and PCF biorepository, in which clinical data, EUS morphology of the cyst, PCF analysis including CEA and amylase, clinical decision, and follow-up were prospectively collected and recorded. All patients gave informed consent for EUS-FNA, standard PCF analysis and remnant volume storage. Immediately after FNA, the PCF was collected into a sterile dry tube that was put on ice and sent to the cytology lab, to be centrifuged for 10 minutes at 2000g, for cytospin preparation for cytology. There supernatant was separated into two samples: Sample A (0.5 mL) was sent to laboratory for routine analysis with CEA, amylase, and cytology in cytospin (performed by experienced cytopathologists); Sample B (remnant PCF) was stored, no more than 30 minutes after collection, in 0.25 mL aliquots at -80˚C until further analysis, in order to minimize thawing and refreezing cycles and stabilize PCF analytes. The amount of PCF stored per patient was variable, according to remnant after standard analysis.

The study cohort consisted of a selected group of 82 patients with pancreatic cysts. The main criterion for patient selection was having more than 1mL of frozen PCF, in either surgical (definitive surgical pathology) or clinicopathological cohorts [EUS-FNA with PCF analysis, with CEA ± diagnostic cytology, and documented stability after prolonged (>24 months) surveillance].

Additionally, in some patients, PCF had been sent for laboratory evaluation of glucose (n=19) and the same type of glucometer “on site” evaluation at time of EUS-FNA (n=7). These

results were retrospectively retrieved from electronic medical records, with the purpose of evaluating the reproducibility of glucose measurement in the frozen samples of PCF.

Standard EUS imaging and PCF analysis with CEA and cytology

EUS findings, including cystic size, location, morphology (thick septs, mural nodules, wall thickening or mass), and main pancreatic duct features (dilatation >10 mm or cyst communication) were retrieved from our database of prospective collected data. In all 82 samples, PCF was evaluated for CEA (Architect, Abbott; chemiluminescent immunoassay) and amylase (Architect, Abbott; kinetic colorimetric method), with CEA (ng/mL) and amylase (UI/L) values available for 78/82 PCLs.

A CEA level greater than 192 ng/mL prompted a classification of a mucinous cyst and lower than 192 ng/mL of a non-mucinous cyst. Cytological analysis of PCF sub-classified the cysts into different types, and (in the current study) for acellular samples the cyst was classified as indeterminate. The reference standard for glucose accuracy analysis was histopathology in the surgical cohort and a definitive cytology in the clinical cohort.

After undergoing EUS-FNA, patients were referred for surgery (surgical cohort) or surveillance, palliation, or endoscopic drainage (non-surgical cohort).

Glucose assay

For this study we evaluated glucose level with a standard glucometer, the Verio One Touch IQ glucometer (LifeScan Europe, Switzerland) [16] that was also used in previous studies,[14,15] using an aliquot of frozen PCF. To minimize variation in analytes, all samples were processed within 30 minutes of thawing, and 2 µl of cystic fluid were analyzed, by pipetting the PCF onto the side of the testing strip. The OneTouch glucometer measures glucose levels between 20 mg/dL and 600 mg/dL, requiring only 0.4 µl of sample. For numerical analysis, we registered glucose readings <20mg/dL as 19 mg/dL. The person performing the measurements was blinded to the final diagnosis. To ensure reproducibility of our measurements, we compared the glucose levels obtained from the frozen samples of PCF, with the glucose levels obtained in fresh PCF at the time of EUS-FNA evaluated in the laboratory (Architect, Abbott; test range of 5-800mg/dL) (n=19) and/or "on site" using the same glucometer (test range of 20-600 mg/dL) (n=7).

Statistical Analysis

Descriptive data are expressed as mean ± SD, median, and interquartile range. To determine differences between cyst types, the Mann-Whitney U test was used for continuous variables and the Fisher-s exact test for categorical variables.

Receiver operating characteristic (ROC) curves were generated and area under the curve (AUC) was calculated to differentiate mucinous from non-mucinous cysts using CEA, glucose, and the combination of CEA with glucose. Correlations between glucose in frozen PCF and CEA, and between glucose in the frozen PCF and fresh PCF using the glucometer or the laboratory assay, were measured using the Spearman´s rank correlation coefficient. Statistical significance was defined as a p-value < 0.05.

Statistical analysis was performed using SPSS Statistics version 24 (Armonk, NY).

RESULTS

Demographics and cyst characteristics

We included 82 patients with frozen PCF samples, of which 78 had prior CEA and amylase measurements. All samples were assessed for glucose levels (supplementary Table 1).

The patient population was composed predominantly of females (59%); the mean age was 61.3±14.8 years (25-91). Pancreatic cysts were located mainly in the head (53%), body (27%), tail (18%), and multiple locations (2%). The mean size of the cysts was 38.5 ± 20.4 mm (8-100) and 67.1% (55/82) of PCLs were larger than 30 mm.

The cyst types included in our study are represented in the flowchart of Figure 1.

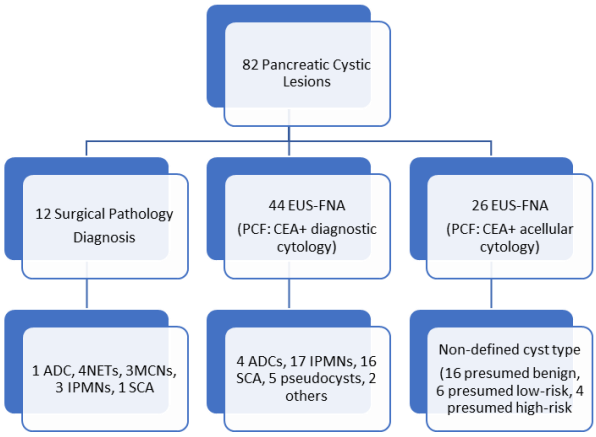


Figure 1. Flowchart with the diagnoses of 82 pancreatic cystic lesions, after surgical pathology or EUS-FNA (CEA ± cytology) and prolonged follow-up.
PCF, pancreatic cyst fluid; ADCs, adenocarcinomas; NETs, neuroendocrine tumors; MCNs, mucinous cystic neoplasms; IPMNs, intraductal papillary mucinous neoplasms; SCAs, serous cystadenomas; low-risk lesion stands for CEA >192 mg/dL and no imaging worrisome features (neither nodule, mass, nor Wirsung dilatation); high-risk lesion stands for imaging worrisome feature (mural nodule, mass or Wirsung >5mm) independently of CEA level.

There were 56/82 lesions with surgical (n=12) or EUS-FNA conclusive cytology (n=44), including 17 SCAs, 5 pseudocysts, 20 IPMNs, 3 MCNs, 5 ADCs, 4 NETs, and 2 other very rare cyst types (1 lymphangioma and 1 acinar cell carcinoma). There were 26 lesions, in which, after PCF analysis, the CEA level obtained, simultaneously, with a non-conclusive cytology, precluded the possibility of cyst type classification. The descriptive data of these 26 samples were analyzed but not included in diagnostic accuracy analysis.

By means of a cut-off value of 192 mg/dL for CEA level, PCLs with conclusive cytopathological diagnoses (n=56) were further classified as: a) Mucinous cysts (n=28), including 20 IPMNs, 3 MCNs, and 5 ADCs; and b) Non-mucinous cysts (n=28), including 17 SCAs, 5 pseudocysts, 4 NETs, and 2 other cyst types.

Table 1 shows clinical, endosonographic and biochemical PCF data of all 82 patients with frozen samples evaluated for glucose level using the glucometer. Patients' gender, cyst size, and cyst location did not differ between mucinous and non-mucinous PCLs, but patients with mucinous cysts were older

(p=0.018), had symptomatic cysts more frequently (p=0.032), and presented mural nodules more often (p=0.020).

Table 1. Demographic and cyst characteristics in mucinous vs. non-mucinous cysts			
	Mucinous (n=38)	**Non-mucinous (n=44)**	***p* value**
Female n (%)	20 (52.6%)	28 (63.6%)	0.372
Mean age ± SD (range)	65.3±13.2 (33-81)	57.3±15.4 (25-91)	0.018
Symptoms* n (%)	10 (26.3%)	1 (2.3%)	0.032
Cyst location (head, body, tail, multiple)	20/9/8/1	23/13/7/1	0.873
Cyst size(mm) mean ± SD (range)	36.7±19.2 (11-100)	40.1±21.5 (16-100)	0.533
Septa n (%)	23 (60.5%)	30 (68.2%)	0.496
Nodule n (%)	14 (36.8%)	6 (14%)	0.020
Adenopathy n (%)	3 (7.9%)	0 (0%)	0.095
Amylase (U/L) median (IQR)	3986 (141-12689)	124 (47-2174)	0.021
CEA (ng/mL) median (IQR)	525.5 (128-7391)	9 (5-20.5)	0.000
Glucose† (mg/dL) median (IQR)	19 (14.3-25)	99 (59-123.3)	0.000
Glucometer reading error	9 (23.7%)	0 (0%)	0.001
Conclusive cytology n (%)	21 (58%)	27(61%)	0.822
IQR, Interquartile range; * pain, weight loss, vomiting; † glucometer assay performed in frozen PCF samples			

Concerning PCF analysis, mucinous cysts had higher amylase and CEA levels (p=0.021 and p<0.0001, respectively) and lower glucose (p<0.0001), but no significant difference was detected in cytological diagnosis (p=0.822) (Table 1).

CEA and Glucose assays

Table 2A shows CEA and glucose levels for mucinous and non-mucinous lesions in which we included only the 56 patients with a definitive pathological or cytological diagnosis. Median CEA was 741 ng/mL (IQR: 165-28566.5) for mucinous and 9 ng/mL (IQR: 5-18.5) for non-mucinous cysts (p<0.0001). Median glucose was <20 mg/dL [(numerical analysis: 19 mg/dL (IQR: 19-19)] in mucinous cysts and 105 mg/dL (IQR: 96-127) in non-mucinous cysts (p<0.0001).

Table 2. Glucose and CEA analysis in pancreatic cystic fluid (surgical and clinicopathologic diagnosis)

(A) Differentiation of Mucinous (n=28) vs all other cysts (n=28)

	Mucinous		**Non-Mucinous**		**P-value**
	Median	**IQR**	**Median**	**IQR**	
CEA (ng/mL)	741.0	165.0-28566.5	9.0	5.0-18.5	<0.0001
Glucose (mg/dL)	19.0	19.0-19.0	105.0	96.0-127.0	<0.0001

(B) ROC curve analysis of tumor markers in cyst fluids for diagnosis of Mucinous pancreatic cysts (n=28 vs n=28)

	AUC	**95% CI**	**P-value**	**Cutoff**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
CEA	O.842	(0.726-0.959)	<0.0001	>192	72 (51-88)	96 (82-100)
Glucose	0.860	(0.748-0.973)	<0.0001	<50	89 (72-98)	86 (67-96)
CEA or glucose	0.853	(0.724-0.963)	<0.0001	>192,<50	88 (70-98)	79 (59-92)

For mucinous cyst diagnosis, CEA with the standard threshold (>192 ng/mL) had a sensitivity and specificity of 72% (95%CI: 51-88) and 96% (95%CI: 82-100), respectively, and the ROC analysis revealed an area under the curve (AUC) of 0.842 (95%CI: 0.726-0.959), as reported in Table 2A.

Glucose level in PCF, with a threshold <50 mg/dL, had a sensitivity of 89% (95% CI: 72-98), a specificity of 86% (95% CI: 67-96) and an AUC of 0.86 (95%CI 0.748-0.973) for mucinous cyst diagnosis, as is reported in Table 2B.

A subgroup analysis of 12 resected cysts, with a surgically confirmed final diagnosis, showed an AUC of glucose that was marginally lower than the AUC of CEA, 0.972 (95%CI 0.727-1) and 1 (95%CI 1-1), respectively (Table 3).

Table 3. Glucose and CEA analysis in pancreatic cystic fluid (surgical diagnosis)

(A) Differentiation of Mucinous (n=6) vs all other cysts (n=6)

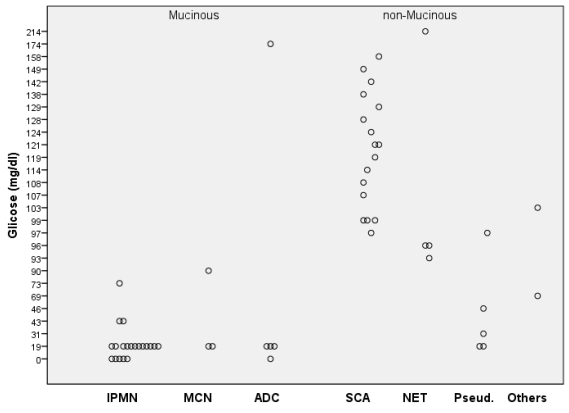
	Mucinous		Non-Mucinous		P-value
	Median	**IQR**	**Median**	**IQR**	
CEA (ng/mL)	2600	808.5-186672.0	11.5	6.88-92.0	0.01
Glucose (mg/dL)	19.0	14.3-36.8	96.0	87.0-127.8	0.006

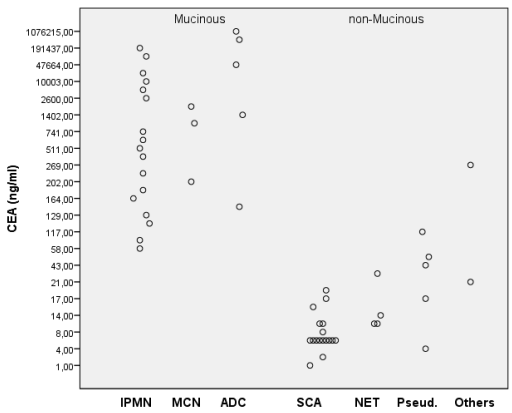
(B) ROC curve analysis of tumor markers in cyst fluids for diagnosis of Mucinous pancreatic cysts (n=6 vs n=6)

	AUC	**95% CI**	**P-value**	**Cutoff**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
CEA	1	(1-1)	0.006	>192	100 (48-100)	83 (36-100)
Glucose	0.972	(0.727-1)	0.022	<50	83 (36-100)	100 (54-100)
CEA or glucose	1	(1-1)	0.004	>192,<50	100 (59-100)	80 (28-99)

A scatter plot dividing mucinous and non-mucinous categories into different cyst types included in each category, with glucose and CEA levels, is in Figure 2A for all 56 cysts with a definitive diagnosis and in Figure 2B for the subgroup of 12 cysts with a surgical pathology diagnosis.

A





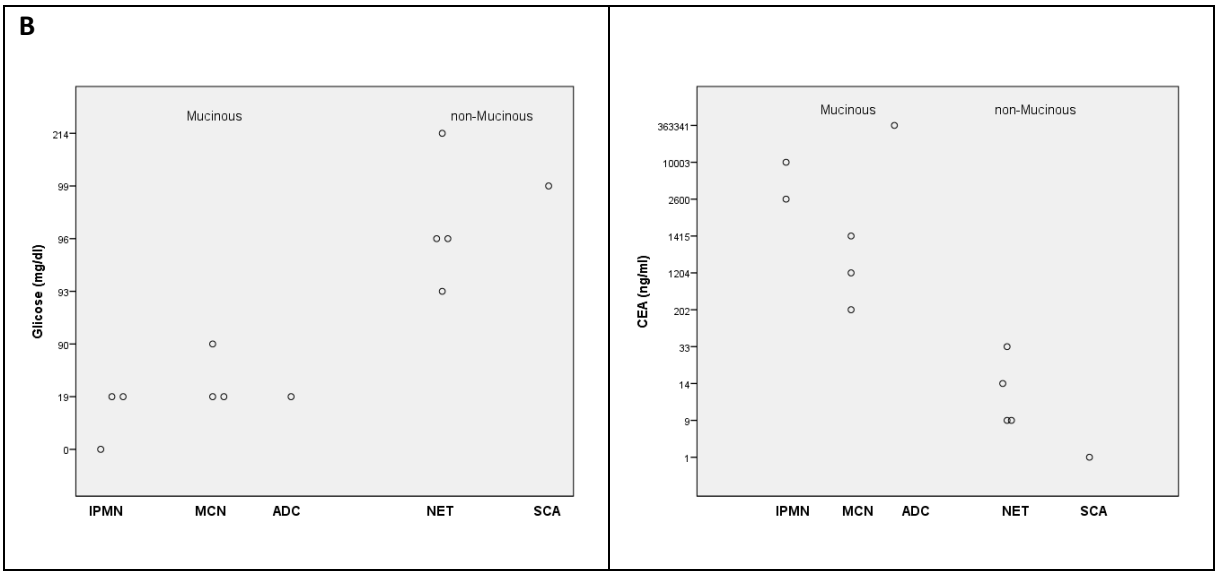


Figure 2. Scatter plot displaying glucose (mg/dL) and CEA (ng/mL) levels in 56 mucinous and non-mucinous pancreatic cystic lesions including both surgical and clinicopathologic diagnosis (A), and only in 12 cystic lesions with a surgical pathology diagnosis (B).
IPMNs, intraductal papillary mucinous neoplasms; MCN, mucinous cystic neoplasm; ADC, adenocarcinoma; SCA, serous cystadenoma; NET, neuroendocrine tumor; pseud, pseudocysts; others, lymphangioma and acinar cell carcinoma.

The highest glucose reading obtained was 214 mg/dL in a non-mucinous cyst, a cystic NET, with all 17 SCAs with glucose levels above 95 mg/dL. Concerning lower glucose readings, there were 19 samples with glucose <20mg/dL, including 12 IPMNs, 2 MCNs, 3 ADCs, and 2 pseudocysts.

Additionally, we registered a reading error of the glucometer in 9/82 (10.9%) of PCF samples, all corresponding to mucinous cysts 9/38 (23.7%) as seen in Table 1. In these cases, high PCF viscosity precluded glucose reading using the glucometer.

Table 1. Demographic and cyst characteristics in mucinous vs. non-mucinous cysts

	Mucinous (n=38)	**Non-mucinous (n=44)**	***p* value**
Female n (%)	20 (52.6%)	28 (63.6%)	0.372
Mean age ± SD (range)	65.3±13.2 (33-81)	57.3±15.4 (25-91)	0.018
Symptoms* n (%)	10 (26.3%)	1 (2.3%)	0.032
Cyst location (head, body, tail, multiple)	20/9/8/1	23/13/7/1	0.873
Cyst size(mm) mean ± SD (range)	36.7±19.2 (11-100)	40.1±21.5 (16-100)	0.533
Septa n (%)	23 (60.5%)	30 (68.2%)	0.496
Nodule n (%)	14 (36.8%)	6 (14%)	0.020
Adenopathy n (%)	3 (7.9%)	0 (0%)	0.095
Amylase (U/L) median (IQR)	3986 (141-12689)	124 (47-2174)	0.021
CEA (ng/mL) median (IQR)	525.5 (128-7391)	9 (5-20.5)	0.000
Glucose† (mg/dL) median (IQR)	19 (14.3-25)	99 (59-123.3)	0.000
Glucometer reading error	9 (23.7%)	0 (0%)	0.001
Conclusive cytology n (%)	21 (58%)	27(61%)	0.822
IQR, Interquartile range; * pain, weight loss, vomiting; † glucometer assay performed in frozen PCF samples			

The association of CEA and glucose levels did not significantly increase the differentiation between mucinous and non-mucinous cysts, with an increase of sensitivity but a reduction of specificity, as presented in Table 2.

Correlation of Glucose and CEA assays

Table 4 shows the results of 19/82 patients, with glucose levels measured in fresh PCF, either with the glucometer at the time of EUS (n=7) or in the laboratory assay (n=19), which were compared to glucose levels measured *a posteriori* in the 82 frozen samples for this study.

Table 4. Glucometer assay in PCF frozen samples, glucometer in EUS-FNA room, laboratory glucose, and CEA at time of EUS-FNA.

Patients	Glucometer assay (Frozen samples) *	Glucometer assay (EUS-room)*	Laboratory Glucose*	CEA (ng/mL)
1	107	133	103	8
2	96		85	33
3	19		4	43
4	114		111	3
5	99		101	5
6	158	145	112	5
7	97		84	17
8	98	122	87	5
9	121		106	9
10	119	114	109	9
11	0	19	5	112
12	0		5	48
13	0		49	511
14	19		4	166
15	19		4	540
16	73		68	87
17	43		38	306
18	19	19	4	47664
19	19	19	4	129

*Glucose (mg/dL); 0- reading error of glucometer; 4 (glucose <5mg/dL); 19 (glucose <20 mg/dL)

The results of glucose are highly reproducible using either technique, in fresh or frozen samples, except for marginally lower levels in laboratory evaluation, but without any change in cyst classification. In fact, there was a strong positive correlation between glucometer-assay in frozen PCF and in-room fresh PCF (r_S = 0.860 and p<0.013), as well as with laboratory glucose in fresh PCF (r_S = 0.874 and p<0.0001).

Analyzing the association of CEA and glucose levels, there was a strong negative correlation between CEA and glucometer-assay for glucose in frozen PCF (r_S = -0.668 and p<0.0001).

DISCUSSION

In this study we tested the diagnostic accuracy of glucose levels on PCF samples obtained by EUS-FNA for pancreatic mucinous lesions and compared it to laboratory CEA level. We showed that by using a standard glucometer for cyst fluid analysis, a low glucose level (< 50 mg/dL) had the same

predictive accuracy as an elevated CEA (>192ng/mL) for mucinous cyst diagnosis. Therefore, the excellent diagnostic performance with immediate on-site result, the low volume required, and the low cost, make glucose assay using a glucometer an excellent biomarker for triage and diagnosis of mucinous cysts.

This study confirms two previous studies that reported high sensitivities, of 92% and 88%, respectively of glucose measurement with a glucometer for mucinous cyst diagnosis, as compared to CEA measurement, which showed a substantially lower sensitivity, of 58% and 77% respectively.[14,15] This high sensitivity is clinically relevant, as a diagnostic test with higher sensitivity is more adequate to diagnose the largest number of lesions as possible. Using CEA level >192 ng/mL as the criterion to diagnose mucinous cysts would exclude several lesions from surveillance, due to reduced sensitivity. In fact, in another previous study, the sensitivity of CEA was only 61%, with 39% of mucinous cysts misdiagnosed using CEA level alone. [7] This imperfect performance of CEA in mucinous cyst diagnosis, may represent a lost opportunity for early diagnosis of pancreatic adenocarcinoma.

In our series, sensitivity of glucose and CEA for diagnosis of mucinous lesions was 89% and 72%, respectively and specificity was 86% and 96%, respectively. Besides its higher sensitivity, the main advantages of glucose are its lower cost, on site availability, and especially the fact that it requires such a small amount of fluid, less than 2 µl. This compares to higher cost and logistical issues of laboratorial glucose and CEA assays, which require 50 µl and 200 µl of sample, respectively. Often, in clinical practice low PCF volume precludes standard biochemical analysis, making the small amount of PCF required for glucometer analysis a major advantage. Furthermore, glucose measurement might be particularly useful in PCLs with CEA levels between 5 and 192 ng/mL and non-diagnostic cytology. In our series this represented 34.1% (28/82) of samples that could have been erroneously classified as non-mucinous and excluded from the surveillance program.

It is also worth noting that we had nine reading errors of glucometer due to high-viscosity, corresponding to almost a quarter of the mucinous samples, and occurring exclusively in mucinous cysts. This has not been reported previously but might become an increasing finding with more frequent use of this technique, occurring due to increased PCF viscosity in mucinous cysts. In our opinion, this should not be a problem, as the so-called string sign has been shown to have a positive predictive value of 94% for diagnosis of mucinous cysts in a study by Bick et al.[17]

As mentioned above, there are only two previously published studies showing that cyst fluid glucose has significant advantages over CEA and should be considered a routine diagnostic test for pancreatic mucinous cysts.[14,15] However, in both studies, most patients were operated on, which might not reflect real life practice, in which most PCLs do not require surgery. To our knowledge, this is the first study to include exclusively EUS-FNA obtained PCF samples, with most patients not being referred for surgery. However, a definitive cytological diagnosis was still available in 56/82 patients and all patients had a follow-up period greater than two years, which strongly supports the non-malignant nature of the lesions studied.

The AUC of glucose for mucinous cyst diagnosis was 0.86 in the 56 patients with a final surgical or clinicopathological diagnosis, and 0.97 in the 12 patients with a surgical diagnosis, corroborating previous studies describing AUC of 0.88,[13] 0.89,[14] and 0.91[15]. The lower AUC of glucose in clinical, compared to surgical series, may be due to a contamination of our clinical series by pseudocysts having a low glucose level similar to mucinous lesions, and rarely require surgery.

However, in clinical practice pseudocysts are rare, and due to clinical context, rarely raise diagnostic issues with other PCLs. In an earlier study low glucose level was also reported in the 6 pseudocysts included, with a median glucose level of 42 mg/dL, the lowest of benign PCLs.[14] In this particular clinical scenario, the EUS findings and CEA level can be complementary to glucose, allowing the diagnosis. On the other hand, glucose has some potential advantages over CEA as a biomarker. Its reproducibility is probably greater than CEA, as the latter optimal cutoff varies with different analyzers. In contrast, glucose levels measured either with glucometer or laboratory assay, in fresh or frozen samples, were highly reproducible, without any change in cyst classification from mucinous to non-mucinous. A limitation common to both biomarkers (glucose and CEA) is the ability to differentiate mucinous cysts but not malignant lesions requiring surgery.

Our study has several strengths. Its main strength is to analyze glucose level in PCF obtained pre-operatively, by EUS-FNA, as is standard in clinical practice. While previous studies included mainly surgically treated patients, in our study the diagnoses are predominantly clinicopathological, which certainly better represents daily clinical practice. Additionally, all data of patients were prospectively collected and registered, except for laboratory and "in room" glucose levels that were retrieved from medical records, resulting in a significant proportion of patients, 95% (78/82), with simultaneous glucose and CEA evaluation. Identically to a previous study,[14] the simultaneous evaluation did not significantly increase the identification of mucinous cysts.

The limitations of this study include the modest sample size and the restricted number of surgical pathology diagnoses (12 out of 82 PCLs), with most diagnoses relying on clinicocytological features, possibly with diagnostic uncertainty. Nevertheless, this type of diagnosis with prolonged surveillance better reproduces clinical practice, in which non-surgical PCLs are predominant, while minimizing possible diagnostic imprecision. Our protocol of PCF storage minimizes pre-centrifugation processing delays and sample delay at room temperature, but even so, glucose is among the most labile metabolites [18] and the readings in frozen samples could have been affected, with consequent heterogeneity in data. However, this was hardly the case, as we found a strong positive correlation between glucometer-assay in frozen PCF and in-room fresh PCF, as well as with laboratory glucose in fresh PCF. Finally, the limited number of PCLs excluded the evaluation of glucose in more pseudocysts and other rare PCLs, such as solid-pseudopapillary neoplasm, among others.

In conclusion, our study demonstrates that glucose level measured by a current glucometer is accurate for mucinous cyst diagnosis. Mucinous cysts present significantly lower glucose level than non-mucinous cysts, and almost a quarter of the mucinous samples displayed a reading error with the glucometer, due to increased viscosity, which itself also points to the diagnosis. Pseudocysts were found to be an exception, with low glucose levels, although being non-mucinous cystic lesions. These results suggest that in clinical practice, on site measurement of glucose at time of EUS-FNA, using a standard glucometer is a powerful tool that may complement or even replace CEA in mucinous cysts diagnosis, especially in small lesions with a limited amount of PCF. Nevertheless, before we accept these results as practice changing, larger prospective studies are needed.

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3. Biomarkers for Diagnosis of High-risk and Malignant Pancreatic Cysts

3.1. A second EUS-FNA for cytology identifies high-risk pancreatic cysts overlooked by current guidelines. Accepted for publication.

Abstract

Background: Endoscopic ultrasound with fine-needle aspiration (EUS-FNA) is recommended for diagnosis of pancreatic cystic lesions (PCLs). Its role in surveillance is unclear. Our goal was to determine if a second EUS-FNA changes diagnosis or management of PCLs.

Methods: A retrospective analysis of an EUS database, searching for EUS-FNAs in PCLs from 2007-2017 was performed. Demographics, cyst characteristics, and FNA results were compared in patients under surveillance, performing a single or two consecutive EUS-FNAs.

Results: Of 203 PCLs referred for EUS-FNA, surveillance was decided in 128 (63%). Data of 105 (82%) patients with a single EUS-FNA were compared with 23 (18%) with two EUS-FNAs during surveillance. Patients were younger in this latter group (p=0.055), whereas CEA levels were marginally higher (p=0.078) and a mass/nodule were more frequent (p=0.006). The mean time between EUS-FNAs was 38 months (4.7-118.8) for 18 patients maintaining surveillance *vs.* 18 months (2.9-56.9) in the four referred for surgery (p=NS) after two EUS-FNAs [2 NETs, 1 IPMN-HGD and 1 MCN-LG]. A high correlation in CEA level between consecutive EUS-FNAs ($r2=0.945$, $p<0.01$) was present, with a change of category observed (cut-off level=192ng/mL) in two patients only. Of four patients with a second EUS-FNA with conclusive cytology, two had NETs confirmed on resection.

Conclusions: Repeating EUS-FNA in surveillance of PCLs with clinical suspicion of malignancy increased neoplasm diagnoses, changing decision toward surgery in almost 20% of patients while excluding IPMNs with mucin nodules from unnecessary resections. A second EUS-FNA for cytology appears justified in some PCLs, particularly for diagnosing NETs.

Key Words

CEA; Cytology; Endoscopic Ultrasound; Fine-needle aspiration; Pancreatic cystic neoplasm

Text

1. INTRODUCTION

Pancreatic cancer is currently the fourth leading cause of cancer death in the USA and it is expected to be the second by 2030. [1,2] Intraductal papillary mucinous neoplasms (IPMNs), are the most frequent pancreatic cystic lesions (PCLs) and represent an excellent opportunity for early diagnosis of pancreatic cancer. [3] PCLs include a wide spectrum of lesions including benign cysts (BCs), pre-malignant cysts (PMCs), and malignant cysts (MCs). The BCs and inflammatory cysts (serous cystadenomas-SCAs, lymphangiomas, pseudocysts) do not progress to malignancy and require, at the most, conservative follow-up. Low-risk PMCs (IPMNs and mucinous cystic neoplasms - MCNs with low-grade atypia) require surveillance due to risk of malignant transformation. For high-risk cysts (IPMNs and MCNs with high-grade atypia) and MCs (adenocarcinomas-ADCs, IPMNs, and MCNs with invasive carcinoma-IC) surgery is recommended. Low grade atypia (LG) includes low and intermediate-grade dysplasia, with cyst fluid aspirates characteristically presenting single, small groups, or flat sheets of cells with atypical cytoplasmic mucin and basally located nuclei.[4] High-grade atypia (HG) comprises high-grade dysplasia

and invasive carcinoma, and presents three main cytological features supporting the diagnosis, background necrosis, chromatin pattern changes and increased nuclear-to-cytoplasmatic ratio.[5] In addition, some rare non-mucinous cysts, including cystic neuroendocrine tumors (NETs) and solid pseudopapillary neoplasms (SPPN), are low-grade malignancies with malignant potential that ordinarily require surgery.

Cross-sectional imaging is inaccurate in distinguishing different PCLs.[6] In current clinical practice, and in contrast with previous guidelines [7], EUS-FNA is recommended for initial diagnosis of undetermined PCLs due to its improved accuracy.[8,9] Carcinoembryonic antigen (CEA) level in pancreatic cystic fluid (PCF) is still considered the most accurate marker for identifying mucinous cysts. [10] Additionally, cytology, despite its limited sensitivity [11], is the only definitive test to allow the diagnosis of malignant PCLs prior to surgical resection.

More recently a combination of molecular markers in PCF and clinical features has shown promising results in classification [12] and in predicting the grade of dysplasia of PCLs [13,14].

Current guidelines for management of PCLs, including the revised Fukuoka guidelines[15], recommend EUS-FNA for diagnosis of PCLs in centers with experience in performing EUS-FNA and interpreting the results, and the AGA guidelines recommend EUS-FNA only in PCLs with at least 2 high-risk features such as size ≥3 cm, a dilated main pancreatic duct, or an associated solid component [16]. Neither of these guidelines endorses repeating the EUS-FNA procedure for resampling cystic lesions with uncertain diagnosis during surveillance. Two previous studies found spurious CEA level fluctuations in consecutive EUS-FNAs, but the value of cytology for diagnosis and clinical decision was not evaluated. [17,18]

The aim of this retrospective cohort study was to determine the added value of a second EUS-FNA with PCF sent for CEA and cytology in PCLs under surveillance, and its impact on cyst classification and clinical decision.

2. MATERIAL AND METHODS

This single-center retrospective study had the approval of the institutional Scientific Board and the Ethics Committee. Consecutive patients with PCLs submitted to EUS-FNA between 2007 and 2017 were selected from a prospectively maintained EUS database. All patients gave informed consent for EUS-FNA, standard PCF analysis, and residual PCF storage.

EUS still-images of all individual case reports were reviewed, and cyst morphology, ductal communication, and main pancreatic duct size were documented. EUS-FNA was performed using a 22-gauge needle. Cysts were fully aspirated when possible, and prophylactic intravenous administration of ciprofloxacin during the procedure was followed by five days of oral administration. The PCF obtained was immediately centrifuged for cytospin preparation for cytological analysis, and the supernatant was sent for CEA and amylase determination. Cysts were classified as mucinous or non-mucinous according to CEA level ≥192 ng/mL, or <5 ng/mL respectively, without extracellular mucin or mucinous epithelium in PCF of non-mucinous lesions. The cytological analysis of PCF, using the Papanicolaou Society of Cytopathology Guidelines [19], prompted cyst classification into three groups: MCs, with atypical or malignant cells and other neoplastic cells (NETs or SPPNs); PMCs with mucinous benign epithelia without atypia or with low-grade atypia and acellular PCF with CEA elevation (level ≥192 ng/mL) supporting the diagnosis; BCs with inflammatory cells, neoplastic benign non-mucinous cells, and low CEA (level <5 ng/mL) supporting the diagnosis. In patients with a non-diagnostic cytology, cysts were classified according to CEA, imaging, and a follow-up of at least two years. In patients referred for surgery, the surgical pathology was the reference standard for diagnosis.

Following EUS-FNA patients were referred for surgery or imaging surveillance, according to the consensus guidelines of Sendai 2006 [20] revised in Fukuoka in 2012 [7], or for palliative care/chemotherapy in case of malignant and unresectable cystic neoplasms. In the surveillance group, imaging with magnetic resonance imaging (MRI) or EUS was performed, and the attending physician ordered a second EUS-FNA if there was a clinical suspicion of malignancy, increase in size, new mural nodule, mass, thickened wall, or diagnostic uncertainty precluding clinical decision. For comparison of two consecutive EUS-FNA results, we reviewed in all cases: 1) EUS imaging features (presence of worrisome features (WFs): cyst size >3 cm, mural nodule, main pancreatic duct dilatation of 5-9mm), according to Fukuoka guidelines revisions 2017 [21] and considered high-risk features according to AGA guidelines 2015 [16]; 2) CEA level (ng/mL) in PCF; 3) cytology result in PCF.

To assess the value of a second EUS-FNA in decision-making, we compared the results and clinical decision after one EUS-FNA (single or first of two consecutive procedures) *versus* the results of the second EUS-FNA.

2.1. Statistical Analysis

Continuous variables were reported as mean ± standard deviation (SD) or median and range. Means of two continuous normally distributed variables were compared by independent samples Student's t test. Mann-Whitney U test was used to compare the distributions of two variables not normally distributed. The frequencies of categorical variables were compared using McNemar's test, Pearson's chi-square test, or Fisher´s exact test. Pearson's correlation coefficient was used to assess the strength of association between two continuous variables. A value of $p<0.05$ was considered significant.

Statistical analysis was performed using SPSS Statistics (version 23; IBM Corp, Armonk, NY).

3. RESULTS

3.1. Patients

Between 2007 and 2017, 306 patients with PCLs were evaluated by EUS with additional FNA for PCF analysis performed in 203 patients. Among these 203 first EUS-FNAs, 128 (63%) patients were referred for surveillance, 38 (19%) for surgery, 20 (10%) for palliation/chemotherapy, and 17 (8%) were lost for follow-up. Of the 128 patients referred for surveillance, 105 (82%) were non-invasively followed with EUS or MRI and 23 (18%) had a second EUS-FNA [Figure 1].

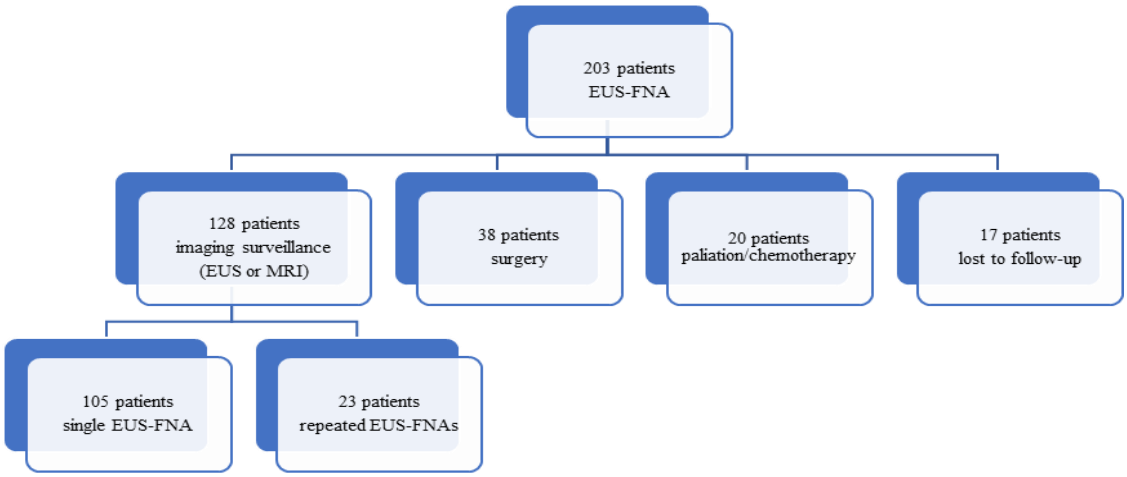


Figure 1. Flowchart with clinical decision in 203 patients with PCNs that performed EUS-FNA.

For the purpose of this study, we compared features of these two groups of patients as shown in Table 1, which summarizes patients' demographics, EUS imaging features, FNA cytology results, and CEA level.

Cysts with two EUS-FNAs occurred in younger patients (60 *vs.* 65 years old; p=0.055), mural nodules or masses were more frequent (p=0.006), and presented a marginally higher CEA level (319 vs 48 ng/mL; p=0.078).

Table 1. Demographics, EUS imaging features, and EUS-FNA results in the first EUS-FNA of patients with two EUS-FNAs (n=23) and patients with a single EUS-FNA (n=105).

	Consecutive EUS-FNAs n=23	Single EUS-FNA n=105	*p*-value
Age, mean (±SD), years	59.52 (13.208)	64,98 (12.038)	0.055[b]
Sex, female/male, %	73.9/26.1	66.7/33.3	0.341 [b]
Head/body/tail/>1 location, %	52.2/34.8/13/0	41.9/38.1/16.2/3.8	0.681 [b]
Diameter, mean (±SD), mm	28.18 (13.211)	29.03 (18.554)	0.839 [b]
HRS-Diameter >3 cm, %	36.4	39	0.507 [b]
Multiple cysts, %	25	24	0.898 [b]
Septa, %	71.4	58.7	0.199 [b]
Mass or nodules, %	40	12.5	0.006 [b]
Adenopathy, %	5	1.9	0.413 [b]
CEA, median (range), ng/mL	319 (0-155012)	48 (1-185614)	0,078[a]
CEA >192 ng/mL, %	53.3	30.9	0,09[b]
Cytology (%) Non-diagnostic Benign Pre-malignant Malignant	 69.6 21.7 0 8.7	 63.1 29.1 1.9 5.8	 0.757 [b]

SD, standard deviation; HRS, high-risk stigmata; a, two-sided p-value; b, one-sided p-value

3.2. Clinical and imaging features in consecutive EUS-FNAs

Cystic morphological features and PCF analysis, including CEA and cytology of the 23 patients with two EUS-FNAs, are summarized in Table 2.

Table 2. Details of consecutive EUS-FNA results and clinical decision in 23 patients with repeated EUS-FNAs.

Patients	Symptoms	Age, years	Gender	EUS-1 Cyst size (mm); nodule; MPD (5-9 mm)*	CEA–1 (ng/mL)	Cytology (PSCG)-1	Clinical Diagnosis-1	Decision-1	Interval between EUS exams (months)	EUS-2 Cyst size (mm); nodule; MPD (5-9 mm)*	CEA–2 (ng/mL)	Cytology (PSCG)-2	Clinical Diagnosis-2	Decision-2	Surgical Pathology
Patient 1	no	72	F	44; no; no	22019	PM	PM	F/U	11	43; no; no	77537	PM	PM	F/U	
Patient 2	no	44	F	25; no; no	2.5	B	B	F/U	15.7	25; no; no	0.5	B	B	F/U	
Patient 3	pain	54	F	45; no; no	519	PM	PM	F/U	4.2	NA; no; no	220	PM	PM	S	MCN-LGD
Patient 4	no	52	F	27; yes; no	1	B	B	F/U	118.8	40; no; no	2.5	B	B	F/U	
Patient 5	no	67	F	12; yes; no	155012	PM	PM	F/U	19.6	12; yes; no	466623	PM	PM	F/U	
Patient 6	no	46	F	16; yes; no	1	B	B	F/U	56.9	16; no; no	3	NO	M	S	NET
Patient 7	no	59	F	40; no; no	1	B	B	F/U	31.7	44; no; no	2	B	B	F/U	
Patient 8	no	33	F	5; yes; no	1594	PM	PM	F/U	12.3	5; yes; no	15	ND	B	F/U	
Patient 9	no	58	F	17; no; no	587	PM	PM	F/U	8.8	16; no; no	1322	PM	PM	F/U	
Patient 10	no	71	F	16; no; no	108	ND	B	F/U	35.7	16; no; no	546	PM	PM	F/U	
Patient 11	dyspepsia	42	F	50; no; no		ND	ND	F/U	118.1	26; no; no		B	B	F/U	
Patient 12	no	33	F	20; no; no	319	PM	PM	F/U	27.4	21; no; no	236	B	PM	F/U	
Patient 13	no	66	F	50; no; no	0.5	B	B	F/U	25.6	50; no; no	0.5	B	B	F/U	
Patient 14	no	65	F	16; no; no	1	B	B	F/U	108.8	32; no; no	1	B	B	F/U	
Patient 15	no	77	F	19; no; no	Insuf	M	PM	F/U	47.3	19; no; no	300754	PM	PM	F/U	
Patient 16	no	62	M	20; no; no	125	ND	B	F/U	18.9	20; no; yes	188	ND	B	F/U	
Patient 17	no	57	M	30; no; no	92	ND	B	F/U	39.2	39; no; no	45	ND	B	F/U	
Patient 18	weight loss	64	M	46; yes; yes		PM	ND	F/U	2.9	46; yes; yes	3198	PM	PM	S	IPMN-HGD
Patient 19	pain	65	M	29; yes; yes	492	PM	PM	F/U	10.6	25; yes; yes		PM	PM	P	
Patient 20	no	55	M	27; yes; no	18	ND	B	F/U	9.5	27; yes; no	9	NO	M	S	NET
Patient 21	no	72	M	19; no; no	102	B	B	F/U	14.7	23; no; no	121	ND	B	F/U	
Patient 22	no	76	F	39; no; no	Insuf	B	B	F/U	4.7	28; no; no	Insuf	ND	ND	F/U	
Patient 23	no	79	F	27; yes; no	1190	PM	PM	F/U	21	27; yes; yes	1273	PM	PM	F/U	

F, female; M, male; non-available, NA; MPD, main pancreatic duct; PSCG, Papanicolaou Society of Cytopathology Guidelines; Insuf, Insufficient; ND, Non-diagnostic; NO, neoplastic other; M, Malignant; B, Benign; PM, Pre-malignant; Ma, Malignant; F/U, Follow-up; S, Surgery; P, Palliation; MCN-LGD, Mucinous cystic neoplasm with low

The global mean time between consecutive EUS-FNAs was 33.2 months (range: 2.9-118.8), with 38 months (range: 4.7-118.8) in the non-surgical group and 18 months (range: 2.9-56.9) in the surgical group of cysts (p=NS).

Only 4 of the 23 patients were symptomatic, all were "non-jaundiced". The 4 patients referred for surgery had final diagnosis of NETs (2), IPMN-HG (1), and MCN-LG (1).

Regarding cyst morphology, 15 and 14 out of 23 patients had WFs in the first and second EUS-FNAs, respectively. In the first, 13 patients had one WF and 2 patients had two WFs, while in the second 11 patients had one WF and 3 had two or more WFs. EUS color Doppler to assess vascularity, revealed non-vascular mural nodules in all instances. Except for 5 patients, mural nodules had <5mm. In patients 5 and 23, nodules were totally aspirated during FNA, confirming they were composed of mucin, patient 19 had a mixed solid and cystic lesion which was referred for palliation, and patients 6 and 20 had the final diagnosis of NETs.

In 6 patients there was increase of cyst size, with a mean of 8 mm (range:1-16 mm), corresponding to a 0.8 mm mean increase in six months, but none of these underwent surgery.

3.3. CEA levels in consecutive EUS-FNAs

CEA level was available in 19/23 patients in the first EUS-FNA, in 20/23 patients in the second, and overall, in 22/23 patients. CEA was ≥192 ng/mL in 8 patients at first EUS-FNA, in 9 at second, and overall, in 11 patients.

Mostly, CEA had minor and non-significant fluctuations between consecutive EUS-FNAs. Only two cysts had a change of classification, one from non-mucinous to mucinous and the other from mucinous to non-mucinous (Figure2).

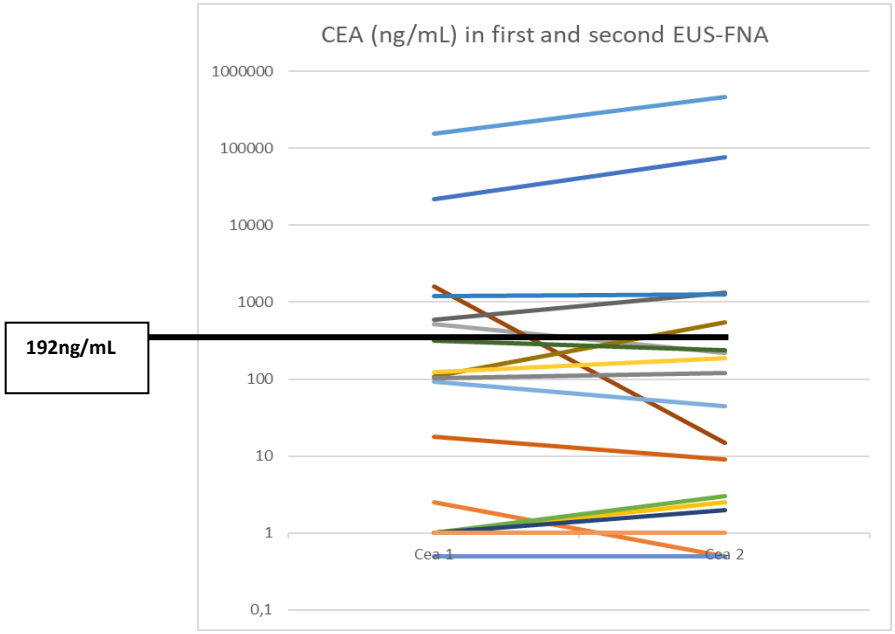


Figure 2. Change in CEA level between two consecutive EUS-FNAs.

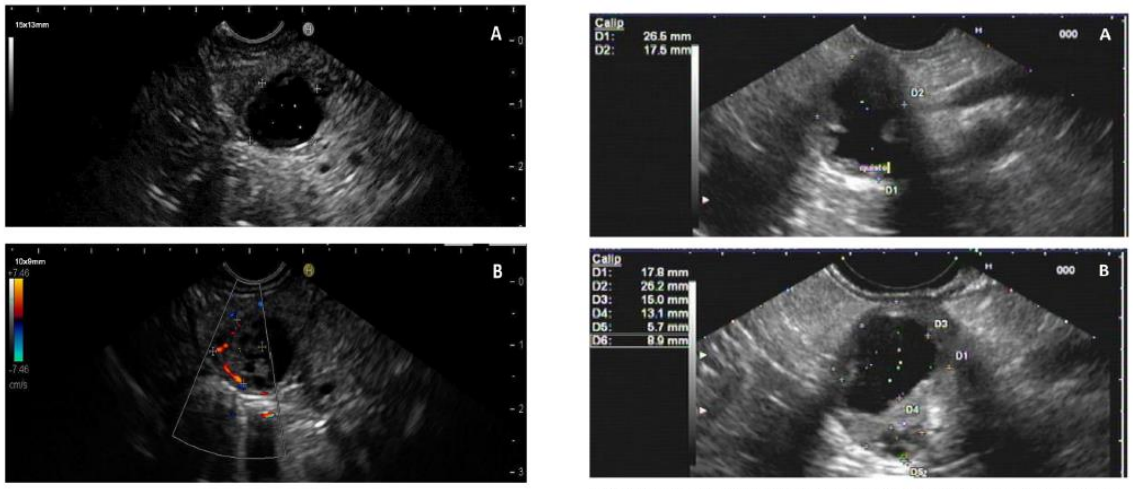
There is a strong positive correlation between CEA levels in consecutive EUS-FNAs after log transformation (r^2=0.945, p <0.01).

3.4. Cytology results in consecutive EUS-FNAs

After two EUS-FNAs, there was 76.1% (35/46) of conclusive cytological diagnoses available, 17 in first and 18 in the second EUS-FNA, there were 2 patients incorrectly diagnosed (2 false negative malignant cases).

In 18 patients with a second EUS-FNA disclosing a conclusive cytology, the diagnosis included: a) 2 NETs identified only after the second EUS-FNA, with the diagnosis confirmed surgically; b) 16 pre-malignant or benign conclusive cytology results, with 1 MCN-LG resected and 13 diagnoses confirmed by prolonged follow-up, including (Patient 15-Table 2) with an atypical cytology in the first EUS-FNA, mucinous benign epithelia in the second, without WFs or morphological changes after surveillance for 84 months. There were 2 false negative results (Patient 18 and 19 - Table 2), the first was resected with an IPMN-HGD in the surgical specimen and the second with an unresectable mucinous carcinoma referred for pain palliation after two consecutive false negative results. Finally, of the remaining 5 patients with a second EUS-FNA with non-diagnostic cytology results, all maintained imaging surveillance, without relevant changes.

In figure 3 we present the EUS images of both NETs. In patient 20, the lesion presented a nodular wall thickening that was more pronounced in the second EUS exam, being described as a mural nodule by the Endosonographer. In patient 6 there was no cyst wall thickening but there was a peripheral nodular area with multiple microcystic spaces. In figure 4, we present the images of EUS-FNA cytology and surgical pathology specimen of patient 18, with the final diagnosis of IPMN-HGD.



Patient 6 – Images of the second EUS exam, with an olygocystic lesion without wall thickening (A) with a peripheral area with multiple microcystic lesions (B).

Patient 20 – Images of the first (A) and second EUS exams (B). There is increase in wall thickening in the size of mural nodules (NET).

Figure 3. EUS images of two neuroendocrine tumours that underwent surgery.

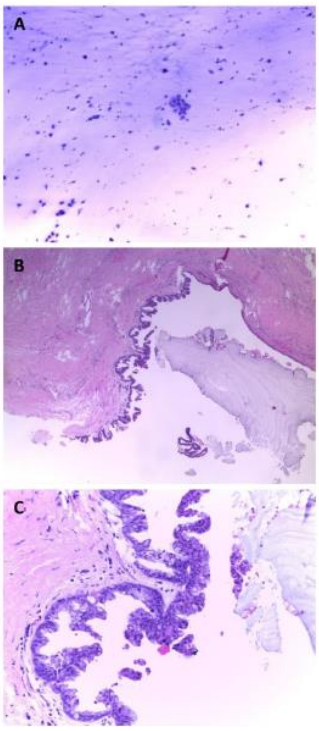


Figure 4. The second EUS-FNA of patient 18 with a diagnosis of a mucinous neoplasm with thick mucus and low-grade cytological atypia (A) and the correspondent surgical pathology specimen of an IPMN with focal high-grade dysplasia (B+C).

3.5. Clinical decision after repeating EUS-FNA

Overall, there was a clinically important change in decision-making following the second EUS-FNA in 4 of 23 patients, from surveillance to surgery (2 NETs, 1 IPMN-HGD, and 1 MCN), as shown in Table 2. Among the remaining 19 patients, clinical decision was to maintain imaging surveillance in 18 cases, without diagnoses of malignancy on follow-up (even in Patient 15-Table 2), and pain palliation in 1 patient with a false negative cytology result.

If only PCLs with 2 or more high-risk features (including size >3 cm, mural nodule or thickened cyst wall) were referred for EUS-FNA, as recommended in the 2015 AGA guidelines [16], 3 of the 4 cystic lesions which were resected, including 2 NETs and 1 MCN (Patients 3, 6, and 20-Table 2), wouldn't had EUS-FNA for diagnosis or had been resected. On the other hand, the revised Fukuoka guidelines [21] endorse, in case of a confirmed mural nodule ≥5 mm, surgery referral if clinically appropriate, what could have led to unnecessary surgeries in 2 patients with PMCs presenting mucin nodules that were aspirated on EUS-FNA (Patients 5, and 23-Table 2).

Although the majority of patients retained the initial cyst classification, resampling PCLs during surveillance for cytology diagnosed high-risk lesions without worrisome features requiring surgery, particularly NETs, while excluding IPMNs with mucin nodules from unnecessary resections.

4. DISCUSSION

In this retrospective single-center analysis, we report the utility of repeating EUS-FNA for cytology in the work-up flow of PCLs under surveillance, especially in those with clinical suspicion of malignancy. This strategy resulted in a change of clinical decision toward surgery in 4 of 23 (17.4%) patients. Although it is predominantly non-diagnostic, PCF cytology was important to support surgical decision, with concordant pathology specimens, while preventing surgery in PMCs with mucin nodules.

The clinical impact of a second EUS-FNA in the surveillance of PCLs is poorly defined and is not recommended routinely. As far as we know, only a single study has previously assessed the benefit of repeating EUS-FNA in patients with suspicious PCLs. The study by Nakai *et al*. [17] included 400 patients, 87 of whom repeated the procedure. The authors measured changes in CEA and reported spurious fluctuations in 20% of patients, without significant changes in EUS findings. However, they did not report cytological results. Considering serial CEA measurements, our results are in agreement with this previous study [17], with CEA fluctuations detected, but changing cyst classification in two cases only. Slight CEA fluctuations may be related to mucin clumping or to different IPMNs subtypes, with no clinical relevance. CEA is useful for cyst classification as mucinous but it does not point to malignancy or high-risk atypia, and by itself should not drive surgical decision [9]. Consequently, it cannot *per se* justify repeating the EUS-FNA. According to a recently published by our group, glucose level in PCF during the first EUS-FNA may complement inconclusive cases in which CEA levels fall between 5 and 192 ng/mL [22].

In the present study, whereas CEA level in PCF showed no additional diagnostic value, a second EUS-FNA was useful for re-evaluating sonographic and/or cytological features and changed clinical management in a subset of patients.

In the initial Fukuoka guidelines [7], EUS-FNA was considered investigational for diagnosis and follow-up of pancreatic cysts, and later, the AGA guidelines [16] recommended EUS-FNA for cysts with two WFs or one new WF detected during surveillance. According to the latter guidelines, four second EUS-FNAs would be performed in our series, including 1 IPMN-HG, 1 unresectable mucinous tumor that presented two WFs, and 2 "non-significant" PMCs presenting Wirsung dilation during surveillance. With these guidelines, the diagnosis of both NETs would be missed, due to the typical scant volume and cellularity of EUS-FNA samples, and no specific diagnostic biomarkers available in clinics for NETs. In the revised Fukuoka guidelines [21], cyst size >3 cm, mural nodules <5 mm, and main pancreatic duct 5-9mm are considered WFs, with EUS recommended for additional stratification. According to the 2018 ACG guidelines [9], EUS-FNA is recommended for cyst diagnosis or if a change in clinical management may be expected. In the present series, the latter guidelines are more accurate, as in the 2 NET cases, 1 had

one WF and the other had none, with 1 being diagnosed 56 months after the first EUS-FNA, corresponding to almost five years of follow-up, the maximum recommended by the AGA guidelines to follow stable PCLs.

Additionally, the significant time interval between consecutive EUS-FNAs in our study among patients referred for surgery and those kept under surveillance, emphasizes the need for and value of prolonged surveillance, due to slow-growth of pancreatic cystic neoplasms.

Although no tool currently available is 100% accurate for characterization of PCLs, cytology, when informative, is the best marker of malignancy, even considering the false negative results of patients 18 and 19.

Despite the limited number of patients, it is worth noting that the greatest benefit of a second EUS-FNA was observed in non-mucinous cysts, even without worrisome features. In fact, in 11 cystic lesions with CEA <192ng/mL, an additional cytological evaluation diagnosed 2 NETs, supporting our belief that EUS-FNA is the best diagnostic tool for these lesions [23]. The limited accuracy of cytology in PCLs due to scant cellularity of PCF may be overcome by new techniques, e.g. micro-forceps biopsy, which (it is hoped) will improve the diagnosis [24] and avoid the need for a second EUS-FNA at a later time.

Our study has limitations, especially the small number of second EUS-FNAs procedures due to strict clinical/imaging criteria used for repeating the procedure and selection bias due to retrospective design. Additional limitations include the limited number of malignant cysts and definitive surgical diagnoses, as well as cyst classification as mucinous and non-mucinous using the CEA cut-off level of 192 ng/mL, since CEA levels between 5 and 192 may be inconclusive for diagnosis.

In conclusion, our study supports a policy of repeating EUS-FNA in PCLs with clinical suspicion of malignancy. CEA levels tend to remain stable, but cytology of PCF influenced decision toward surgery, with 2 NETs misdiagnosed as SCAs on the first EUS-FNA. Long-term impact of repeating an EUS-FNA particularly in cysts with CEA <192ng/mL deserves further investigation.

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3.2. Endoscopic Ultrasound with Fine Needle Aspiration is useful in pancreatic cysts smaller than 3 cm. Submitted for publication.

Abstract

Background: In current guidelines, endoscopic ultrasound with fine-needle aspiration (EUS-FNA) is recommended in pancreatic cystic lesions (PCLs) with worrisome features (size ≥3 cm, mural nodule, or Wirsung dilation).

Objective: To evaluate EUS-FNA for diagnosis and clinical decision in PCLs smaller than 3 cm.

Material and Methods: Retrospective study of PCLs smaller than 3 cm (2007-2016) undergoing EUS-FNA. Clinical, EUS and pancreatic cystic fluid (PCF) data were prospectively registered. Performance of EUS-FNA with PCF analysis for the detection of malignancy and surgical triage were analyzed.

Results: We evaluated 115 patients with PCLs<3 cm who underwent EUS-FNA. 19 patients underwent surgery, 7 had malignant lesions, 8 pre-malignant lesions, and the remaining 4 benign lesions. Mass/mural nodule was present in 27% of the cysts, CEA level was higher than 192ng/mL in 39.4% of patients, and only 35% of cytologic samples were informative. Nevertheless, biochemichal and cytologic criteria, and FNA for PCF analysis improved the diagnostic performance of EUS imaging - AUC=0.80 vs AUC=60.

Conclusion: EUS-FNA in PCLs<3cm confirmed malignancy, even in lesions without worrisome features (nodule/mass), with two in every five resections showing high-risk/malignant lesions. EUS-FNA was also useful to diagnose benign cysts, allowing surveillance to be stopped in one in every five patients.

Keywords

CEA, pancreatic cyst, EUS-FNA, IPMN, MCN, small size.

Text

INTRODUCTION

Widespread use of abdominal imaging led to a significant increase in the diagnosis of asymptomatic pancreatic cystic lesions (PCLs) [1], including benign/inflammatory, pre-malignant, and malignant cysts.

The key question for pancreatic cyst management is to distinguish patients harboring advanced neoplasia who should be submitted to surgery, from those with pre-malignant lesions who require surveillance, and those with benign lesions who should be released from surveillance programs. In the absence of robust prospective data to answer these questions, current guidelines for management of PCLs are mostly driven by low-quality evidence, consensus, and opinion of experts [2,3,4,5].

Several of these guidelines [2,3,5] provide management guidance for PCLs with emphasis on high-risk features, including size greater than 3 cm, mural nodules, and dilation of the main pancreatic duct. The 2017 revised Fukuoka guidelines [3] propose using a cyst size greater than 3 cm as a worrisome feature to recommend EUS-FNA. The 2015 AGA guidelines [2] include cyst size greater than 3 cm as one of three high-risk features, along with dilation of the main pancreatic duct and presence of a mass or nodule, which should prompt an EUS-FNA, but only if two of these features are present

simultaneously. Thus, in current clinical practice, for cysts greater than 3 cm, EUS-FNA is a reasonable next step.

The described risk of malignancy in cysts larger than 3 cm is about 9% and compares to 6.5% for cysts smaller than 3 cm [6]. As such, current guidelines may be considered too stringent [7,8,9] and the risk of missing malignancy or high-grade dysplasia seems considerable. The superior imaging quality of EUS and additional FNA for pancreatic cyst fluid (PCF) analysis, including carcinoembryonic antigen (CEA) and cytology, may allow definitive cyst classification [10]. CEA allows distinction of mucinous cysts [11] and a cytology of malignancy, which despite scant cellularity [12] and interobserver agreement limitations[13], provides the definitive diagnosis of malignancy.

The aim of our study was to evaluate the added value of EUS-FNA for cystic fluid analysis in the diagnosis of high-risk pancreatic cysts smaller than 3 cm and its potential to change clinical decision.

MATERIALS AND METHODS

Case selection

For this single-center retrospective study, we reviewed consecutive patients with PCLs submitted to EUS between 2007 and 2016, and selected cysts smaller than 3 cm that were further evaluated with FNA, from our Endoscopic Ultrasound database and Pancreatic Cyst Registry, as approved by the Institutional Scientific Board and Ethics Committee (UIC/ 1143). For all patients, clinical data, EUS morphology, PCF analysis (CEA, amylase and cytology), clinical decision, and follow-up were prospectively collected and registered.

All patients gave informed consent for EUS-FNA, standard PCF analysis, and residual PCF storage.

The main criterion for patient selection was having been submitted to EUS-FNA for evaluation of a pancreatic cyst smaller than 3 cm. Patients were divided into a surgical cohort, with definitive surgical pathology as reference standard for diagnosis, and a clinical cohort, with the diagnosis established by EUS-FNA with PCF analysis for CEA and/or cytology and morphologic stability after imaging surveillance for a minimum of six months.

EUS still-images were reviewed, with EUS findings, including cyst size, location, morphology (thick septs, mural nodules, wall thickening, or mass), and main pancreatic duct features (dilation of 5-9 mm or cyst communication) retrieved from our database of prospectively collected data. EUS-FNA was performed using 22 or 25-gauge needles, as per attending physician decision. Cysts were fully aspirated when possible with prophylactic intravenous administration of ciprofloxacin during the procedure, followed by five days of oral administration.

The PCF obtained was immediately centrifuged for cytospin preparation for cytological analysis, and the supernatant was sent for CEA (Architect, Abbott; chemiluminescent immunoassay) and amylase (Architect, Abbott; kinetic colorimetric method). Cysts were classified as mucinous, indeterminate, or non-mucinous according to a CEA level ≥192 ng/mL, between 192ng/mL and 5ng/mL, or <5ng/mL, respectively. Cysts were further classified as benign serous cystadenomas (SCAs), in case of CEA level <5ng/mL with a matched or non-diagnostic cytology. The cytological analysis of PCF, using the Papanicolaou Society of Cytopathology Guidelines [14], prompted cyst classification into three groups: 1) malignant cysts (MCs), having atypical or malignant cells and other neoplastic cells (cystic adenocarcinomas - ADCs, neuroendocrine tumors - NETs or solid

pseudopapillary neoplasms-SPPNs); 2) pre-malignant cysts (PMCs) with mucinous benign epithelia without atypia or with low-grade atypia, including mucinous cystic neoplasms - MCNs and intraductal papillary neoplasms - IPMNs; and 3) benign cysts (BCs) with inflammatory cells, neoplastic benign non-mucinous cells, or other neoplastic cells, for example SCAs, pseudocysts, lymphangiomas.

Clinical Decision

After undergoing EUS-FNA, patients were referred for surgery (surgical cohort) or surveillance, palliation, or endoscopic treatment (clinical cohort), according to the consensus guidelines of Sendai 2006 [15] revised in Fukuoka in 2012 [16] and attending physician's decision. Magnetic resonance imaging (MRI) or EUS were used in surveillance of the clinical cohort.

For comparison of patient and cyst characteristics underlying surgical referral after EUS-FNA, we reviewed: 1) EUS imaging features (WFs other than size: mural nodule or mass, main pancreatic duct dilation of 5-9mm), according to 2017 revised Fukuoka guidelines [3] and considered high-risk features according to 2015 AGA guidelines [2]; 2) CEA level (ng/mL) in PCF; and 3) Cytology result of PCF.

To assess the impact of EUS-FNA in clinical management, we predicted the decision endorsed by current guidelines according to EUS-FNA evaluation (cyst morphology and PCF analysis with CEA and cytology).

Statistical Analysis

Descriptive data were expressed as mean ± SD or median, and range. Chi-square test or Fisher's exact test were used to assess differences between cysts that required surgery and cysts maintained on surveillance, for dichotomous variables, and student t-test or the Wilcoxon rank-sum test for continuous variables. A statistical significance was defined as a p-value < 0.05. The sensitivity, specificity, positive and negative predictive value, and diagnostic accuracy of EUS imaging, CEA level, and cytology in PCF were evaluated for the diagnosis of high-risk/malignant cysts in the surgical cohort. Receiver operator curves were generated, and area under the curve (AUC) was calculated. Statistical analysis was performed using SPSS Statistics version 23 (IBM Corp, Armonk, NY).

RESULTS

Demographics and cyst characteristics

Of 167 patients referred to EUS for evaluation of PCLs smaller than 3 cm, we evaluated 115 who underwent additional FNA for PCF analysis, including surgical and clinical cohorts with 19 and 96 patients, respectively. Table 1 shows clinical, endosonographic, and PCF analysis for all patients included. There were 49/115 (42.6%) PCLs that were 2 cm or more in size and a mural nodule was present in 27% (31/115) of the cysts. There were no patients with Wirsung dilation. No adverse events related to EUS-FNA were recorded in the present series.

Table 1. Demographics, cyst morphological features, and PCF analysis.	
Patients, n	**115**
Females, n (%)	75 (65%)
Mean age, years Mean ± standard deviation (range)	 63 ± 12 (33-86)
F-up time, months Mean ± standard deviation (range)	 37 ± 30 (6-134)
Symptoms, n (%)	21 (18.3%)
Cysts, n	**115**
Location n, (%)	
Head Body Tail Multiple	45 (39.1%) 42 (36.6%) 23 (20%) 5 (4.3%)
Size, mm Mean ± standard deviation (range)	 19± 6 (5-29)
Size, n (%) ≤10 mm 10-20 mm ≥20 mm	 10 (8.7%) 56 (48.7%) 49 (42.6%)
Mural nodule/mass, n (%)	31 (27%)
Wirsung dilation, n (%)	0 (0%)
PCF analysis	
CEA, n (%)* ≤5 ng/mL 5-192ng/mL ≥192 ng/mL	 18 (18.2%) 42 (42.4%) 39 (39.4%)
Amylase, n (%)** <250 UI/mL ≥250 UI/mL	 38 (39.6%) 50 (60.4%)
Cytology Acellular Benign or inflammatory LGD Malignant, atypical, NET	 75 (65.2%) 20 (17.4%) 3 (2.6%) 17 (14.8%)
Clinical decision after EUS-FNA, n (%) Surgery Imaging Surveillance Palliation*** Lost to follow-up	 19 (16.5%) 80 (69.6%) 5 (4.3%) 11(9.6%)
*CEA available in 99 patients; **amylase available in 96 patients; *** bad surgical candidates (2)/unresectable concomitant ADCs (3)	

Combining CEA level and cytology results in PCF, we found that in 18 PCLs with CEA level ≤5 ng/mL, cytology identified 1 malignant, 1 NET, and 3 benign lesions. For the 42 PCLs with a CEA level between 5 and 192ng/mL, cytology identified 2 atypical, 2 NETs, and 6 benign lesions. For the 39 PCLs with a CEA ≥192ng/mL, cytology identified 4 malignant, 7 mucinous (including 3 samples with low-grade atypia), and 6 benign lesions. Considering cytology as the pre-surgical gold standard of malignancy, CEA values had considerable overlap in malignant and non-malignant cysts, without discriminative power (p=0.053).

Surgical pathology diagnosis and EUS-FNA diagnostic performance

Figure 1 shows the discriminative power of nodule/mass within the cyst in surgical patients. The overall rate of surgery in patients with cysts smaller than 3 cm evaluated with FNA was 17% (19/115).

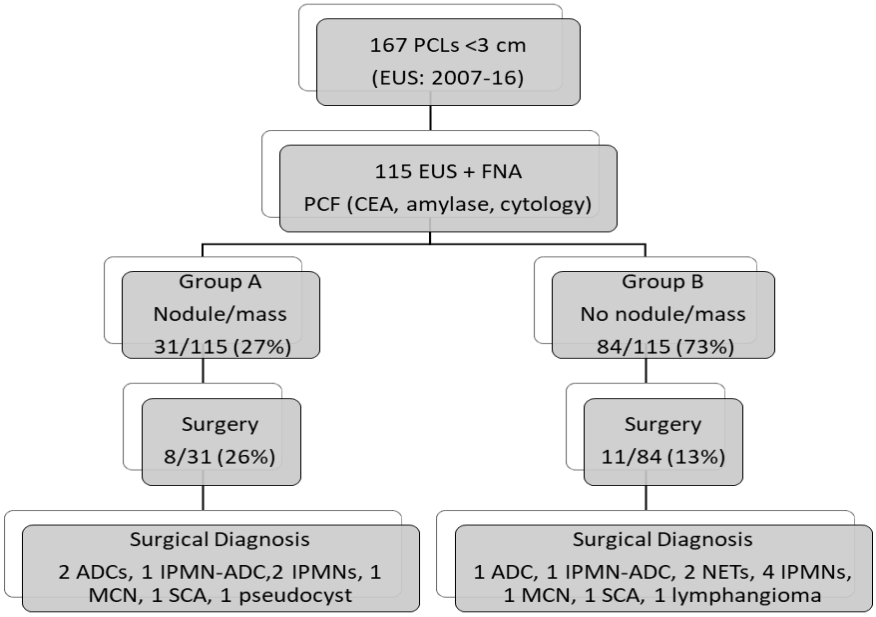

Figure 1. Flowchart with the pancreatic cysts studied by EUS-FNA and the diagnosis of 19 pancreatic cystic lesions that underwent surgery.

In the subgroup of 31 patients with a concomitant mass or nodule (Group A), the rate of surgery was 26% (8/31), while in the subgroup of 84 patients without mural nodule or mass (Group B), the surgery rate was 13% (11/84), *p*-value of 0.092. Detailed data of lesions that underwent surgery are shown in the Supplementary Table. Broadly, 7 malignant, 8 pre-malignant, and 4 benign PCLs were resected. The 7/19 (37%) of malignant or high-risk lesions included cystic PDACs (3), NETs (2), and IPMNs-ADC (2). As shown in Table 2, the accuracy of EUS imaging was improved by PCF analysis for cytological diagnosis of malignant cysts.

Table 2. Performance characteristics of EUS imaging, PCF fluid analysis (CEA and cytology), and EUS-FNA results (imaging, CEA and cytology) for malignant cysts identification.

Malignant cysts (7/19)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)	Area under the ROC (CI)
EUS – Imaging	38 (9-76)	55 (23-83)	33 (14-59)	60 (41-76)	48 (25-72)	0.60 (0.30-0.90)
EUS-FNA (CEA+cytology)	86 (42-99)	50 (21-79)	50 (35-66)	86 (47-98)	63 (38-84)	0.80 (0.58-1.00)

EUS, endoscopic ultrasound; FNA, fine-needle aspiration; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; ROC, receiver-operating characteristics

Table 3 compares demographic, clinical, and cystic features of patients harboring malignant/high risk and non-malignant lesions.

Table 3. Demographics and cystic features in malignant vs. non-malignant cysts in both cohorts.

	Malignant (n=17)	**Non-malignant (n=98)**	***p* value**
Female n (%)	9 (52.6%)	65 (66.3%)	0.551
Mean age ± SD (range)	62.9±12.3 (43-80)	63.1±11.9 (33-86)	0.987
Symptoms* n (%)	12 (70.6%)	9 (9.2%)	**0.000**
Cyst location (head, body, tail, multiple)	10/5/2/0	35/37/21/5	0.059
Cyst size (mm) mean ± SD (range)	21.6±6 (10-29)	18±6.1 (5-29)	**0.030**
Cyst size >20 mm	10 (58.8%)	33 (33.7%)	**0.049**
Septa n (%)	6 (35.3%)	56 (57.1%)	0.080
Nodule n (%)	10 (58.8%)	21 (21.4%)	**0.001**
Adenopathy n (%)	6 (35.3%)	1 (1%)	**0.000**
Amylase (U/L) ± SD (range)	3564±10644 (7-40223)	41437±111030 (3-786486)	0.049
CEA (ng/mL) ± SD (range)	1522±39505 (5-150490)	2725±17173 (1-155012)	0.053
Conclusive cytology n (%)	14 (82.4%)	25 (25.8%)	**0.001**

SD, standard deviation; * pain, weight loss, vomiting, jaundice, acute pancreatitis;

Symptomatic lesions, with a larger size and presenting a nodule/mas or suspicious lymph nodes, with a conclusive cytology, were more often malignant. Mural nodules were relevant for diagnosis of mucinous malignant lesions, but not for other rare types of high-risk lesions (e.g. cystic NETs and ADCs), in which a thick wall justified FNA, with cytology rendering the final diagnosis. Although mural nodules correlate with malignancy, they are not pathognomonic, as they also occur in low-risk lesions.

DISCUSSION

Our study endorses EUS-FNA with PCF analysis in cysts smaller than 3 cm, even if additional worrisome features (mural nodule/mass) are absent, due to its ability to diagnose malignant small PCLs pre-operatively. In our series we found malignant and pre-malignant lesion in 15/115 patients (13%), which is similar to the rate of malignancy in lesions greater than 3 cm [17]. Combined EUS-FNA has better performance that isolated EUS imaging for malignant cyst diagnosis, with an area under the curve of 0.8 (95% CI: 0.58-1).

In our cohort of 19 surgical resected cysts, we found 7/19 (37%) histologically high-risk/malignant cysts, including NETs, IPMN-associated ADCs, and cystic ADCs. This rate of malignancy in small cysts is similar to 35.5% of histologically malignant cysts in lesions larger than 3 cm, as reported by Chebib et al. [17] and higher than other surgical series, with 16% of high-risk malignant IPMNs, as reported by Ridtitid et al. [18], 32% by Singhi et al. [8] , and 29% by Lekkerkeker et al. [9]. Furthermore, 8/19 patients had pre-malignant lesions.

Our results support the concept that size by itself should not be a decisive factor to perform or not perform FNA. In order to select high-risk/malignant in our series of small cysts, the presence of a nodule *per se* was not particularly helpful, with a 10% malignancy rate in surgical specimens of PCLs associated with a mural nodule/mass (Group A) *versus* 5% of malignant surgical specimens without nodule/mass (Group B) (NS). This would apparently support the recommendation of AGA guidelines requiring at least two worrisome features for further evaluation of PCLs [2]. However, we had 5% of small cysts without worrisome features that were confirmed to be high-risk or malignant in surgical pathology specimens, including two NETS and one cystic ADC. In a recent meta-analysis that evaluated risk factors for malignancy and high-grade dysplasia in IPMNs, the presence of a nodule was

considered relevant, but not cyst size [19] The inclusion of other relevant PCLs besides IPMNs in our study, particularly two NETs and a cystic ADC, without associated nodules, may possibly explain these apparent discrepancies. Our results are in line with previous publications [8] and expert opinions [20] that AGA guidelines [2] may be imprecise in discriminating between neoplastic and non-neoplastic cysts, and of limited value in early detection of pancreatic cancer. More recent guidelines, recommending EUS-FNA for PCF analysis in indeterminate cysts, are probably more adequate for this purpose [4].

Additionally, in our series there were 18% (18/99) of patients with non-mucinous cysts after EUS-FNA (CEA level ≤5 ng/mL), supporting a strategy to stop surveillance. However, in these 18 patients cytology identified 1 malignant cyst and 1 NET, further reinforcing the value of a conclusive cytology to definitely exclude malignancy. Similarly, there were 42% (42/99) of PCLs with a CEA level between 5 and 192ng/mL, considered indeterminate for mucinous cyst diagnosis, with cytology diagnosing 4 high risk/malignant lesions - 2 atypical and 2 NETs. Using CEA level ≥192 ng/mL as cut-off for mucinous cysts diagnosis would reduce sensitivity and exclude several mucinous lesions from surveillance. This imperfect performance of CEA may represent a lost opportunity for early detection of pancreatic cancer, highlighting the need of better biomarkers in PCLs. For this purpose, PCF glucose may be more advantageous than CEA in routine diagnosis of small pancreatic mucinous cysts, reducing "indeterminate" diagnosis with minimal amount of PCF required as shown by others and ourselves in a recently published study [21,22,23].

Our study has several strengths. The main is to evaluate PCLs assessed by EUS-FNA as standard of care including predominantly low-risk PCLs, better representing daily practice. Moreover, the patient and cyst data were prospectively collected and registered with most PCLs with CEA and cytology evaluation. We had CEA level for most PCLs, but cytology was informative in only 33% of patients, which compares to 76% in lesions larger than 3 cm [17].

The limitations of our study include its retrospective design, which may have introduced unintended biases, the modest sample size, and the low number of surgical pathology diagnoses. Also, we evaluated cyst morphology by EUS but not by other imaging methods. Finally, we studied diagnostic accuracy of EUS-FNA exclusively in resected cysts due to possible diagnostic uncertainty of the clinical cohort, in which diagnosis relied on clinico-cytological features.

In summary, when debating the role of EUS-FNA in pancreatic cysts smaller than 3 cm, one can support either side of the coin. On the one hand, we found most PCF analysis inconclusive (more than 40% of CEA levels between 5 and192ng/mL, and overall, two thirds of acellular samples) making EUS-FNA an invasive and often unhelpful technique. In contrast, EUS-FNA allowed a diagnosis of malignancy in some patients who would otherwise be surveilled, potentially improving outcome and cost-effectiveness of the program. Mass/nodules were helpful for malignant cyst diagnosis, although lacking in cystic NETs and PDACs and occurring in non-high-risk IPMNs. As the performance of any isolated marker is imperfect, according to our results, combining clinical, morphologic, biochemical, and cytological data significantly improves the diagnosis of malignancy. Furthermore, EUS-FNA diagnosed benign cysts in almost 1 in every 5 patients, allowing their release from invasive and costly surveillance programs. Surveillance is especially important in young and healthy patients, while discontinuation is advisable in elderly individuals with increased risk of death from causes other than pancreatic cancer.

In conclusion, EUS-FNA allows pre-operative diagnosis of even small PCLs harboring malignancy. Finding new and more specific biomarkers may also enhance this strategy.

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Supplementary Table. Clinical, imaging, biochemical, cytologic features and final diagnosis of 19 resected cysts.

Pt	Gender, age(y)	Symptoms	EUS: Cyst, nodule(cm)	Imaging: Diagnosis	FNA CEA (ng/mL)	FNA cytology	FNA Diagnosis (CEA+cytology)	Surgical pathology	Final Diagnosis
1	F, 74	New-onset diabetes	2, Un	High-risk	2297	Malignant	Malignant	ADC	Malignant
2	F, 55	Pain	2.5, 4	High-risk	15306	Acelular	Pre-malignant	IPMN-ADC	Malignant
3	F, 47	Pain	1.5, No	Low-risk	24	NET	Malignant	NET	Malignant
4	F, 68	No	2, No	Low-risk	Thick	Acellular	Pre-malignant	IPMN	Pre-malignant
5	M, 72	Jaundice	1.5, No	Low-risk	88	Acellular	Un	ADC	Malignant
6	F, 35	No	1.7, No	Low-risk	45	Lymphocytes	Benign	Lymphangioma	Benign
7	M, 77	Pain	1.5, No	Low-risk	207	Acellular	Pre-malignant	IPMN	Pre-malignant
8	F, 57	Pain	2.3, No	Low-risk	5	NET	Malignant	NET	Malignant
9	M, 75	Pain	2, No	Low-risk	10003	Atypical	Malignant	IPMN-ADC	Malignant
10	M, 49	No	2.5, 1.5	High-risk	19	Acellular	Un	Pseudocyst	Benign
11	F, 56	Pain	1.7, No	Low-risk	1759	Acellular	Pre-malignant	MCN	Pre-malignant
12	F, 37	No	2.4, 0.6	High-risk	305	Acellular	Pre-malignant	MCN	Pre-malignant
13	M, 67	Weight loss	1.5, Un	High-risk	574	Acellular	Pre-malignant	IPMN	Pre-malignant
14	M, 55	No	2.7, 0.9	High-risk	18	Acellular	Un	SCA	Benign
15	F, 51	Pain	2, 2	High-risk	Un*	Atypical	Malignant	IPMN	Pre-malignant
16	F, 64	**No**	2.9, No	Low-risk	1	Malignant	Malignant	SCA	Benign
17	F, 63	No	2.4, No	Low-risk	356	Atypical	Malignant	IPMN	Pre-malignant
18	F, 77	No	1.8, No	Low-risk	999	Mucinous, LGA	Pre-malignant	IPMN	Pre-malignant
19	F, 73	Pain	2.8, 1.2	High-risk	2600	Atypical	Malignant	ADC	Malignant

Pt, patient; F, female; M, male; Un-unknown size;*Thick fluid;

3.3. Chromogranin A and NSE in pancreatic cystic fluid are useful biomarkers for diagnosis of cystic pancreatic neuroendocrine tumors. Submitted for publication.

Abstract

Purpose: Pancreatic cysts are increasing incidental findings that encompass a wide spectrum of lesions, including cystic pancreatic neuroendocrine tumors (cPanNETs). To evaluate Chromogranin A (CroA) and neuron-specific enolase (NSE) levels in pancreatic cystic fluid (PCF) for cPanNETs diagnosis.

Methods: PCF samples were selected from the EUS-FNA registry of our Hospital, which started in 2008 and is used for diagnosis and clinical management of patients with pancreatic cysts. We studied PCF obtained by EUS-FNA from pancreatic cysts with surgical or clinicopathological diagnosis. PCF carcinoembryonic antigen (CEA), amylase, and cytology were available. Glucose (glucometer), CroA (CGA-RIACT®, Cisbio Bioassays, France), and NSE (LIAISON®XL NSE, DiaSorin, Italy) were measured in 0.25 mL of frozen PCF. We studied samples of cPanNETs and other different types of PCLs with more than 1mL of frozen PCF.

Results: Sixteen patients were included in the study, comprising 9 females, with a mean age of 58±12 years (42-79). PCLs were mainly located in the head (7), followed by body (5) and tail (4), with a mean size of 29.3±12.3 mm (15-60), and 31% (5/16) of cysts were larger than 3 cm. The sixteen PCF samples included 4 cPanNETs, 7 benign (4 serous cystadenomas, 3 pseudocysts), 3 pre-malignant (3 IPMNs), and 2 malignant (1 acinar cell tumor, 1 IPMN-HGD) cysts were studied. Amylase, CEA, and glucose levels were not significantly different in cPanNETs. CroA and NSE levels were higher in cPanNET (median values of 319.2 ng/mL and 412.7 ng/mL, respectively) than in non-cPanNETs (median values < 42 ng/mL and <0.8 ng/mL, respectively), p=0.005 and p=0.002, respectively. In the diagnosis of cPanNETs, the AUC was 0.938 (95%CI: 0.81-1) for CroA and 1 (95%CI: 1-1) for NSE, with optimal cut-off values >149 ng/mL for CroA and >99 ng/mL for NSE.

Conclusion: Our preliminary study suggests that elevated CroA and NSE in PCF accurately identify cPanNETs. More extensive research is needed to confirm these findings.

Keywords

Chromogranin A; NSE; Pancreatic cyst; Glucose; EUS-FNA; Pancreatic neuroendocrine tumor

Text

INTRODUCTION

The number of asymptomatic pancreatic cystic lesions (PCLs) is increasing due to the wide use of abdominal imaging[1], with a 2.4% prevalence of cysts in Magnetic Resonance Imaging (MRI)[2]. As most incidental PCLs are neoplastic[3], with more than half being premalignant or malignant[4], they entail significant additional anxiety, tests, and costs[5]. After a benign, pre-malignant, or malignant cyst diagnosis, the clinical recommendation is no follow-up, conservative surveillance, or surgery, in accordance with malignancy risk[3]. Intraductal papillary mucinous neoplasms (IPMNs), mucinous cystic neoplasms (MCNs), and serous cystadenomas (SCAs) are the most frequent types of cysts[6], representing about 90% of all PCLs.

Pancreatic neuroendocrine tumors (PanNETs) are predominantly solid, but cystic lesions have been reported to represent 13% to 19% of all PanNETs[7,8].The biological behavior of cystic PanNETs (cPanNETs) and solid PanNETs (sPanNETs) is similar in terms of malignancy risk[6,8], requiring close surveillance or surgical treatment, according to size. Most cPanNETs are non-functional, typically thick-walled, unilocular, and cannot be differentiated solely by imaging from other cystic lesions, especially if small sized[6,9]. Endoscopic Ultrasound with fine-needle aspiration (EUS-FNA) and cytology has become standard in pre-operative evaluation of PCLs. The sensitivity of EUS-FNA for diagnosis of cPanNETs is 63% to 71%[10]. Cytology is the most accurate test of diagnosis, with high specificity, while CEA and amylase are non-contributory[7].

Although CEA level is significantly lower in PCF of cPanNETs than in mucinous cysts, this is also true for SCAs, one of the most frequent PCLs. The diagnostic yield of cytology in cPanNETs varies from 73% to 90%[11,12], is limited in cases of scant cytology, and a misdiagnosis of benign SCAs may occur. Overall, cytology of PCF is highly specific for malignant PCLs, but one third of cysts can be incorrectly diagnosed pre-operatively[13], and differential diagnosis becomes especially difficult in cases of rare cysts, including cPanNETs. An additional biomarker in PCF for diagnosis of cPanNETs, non-mucinous cysts with malignancy risk, would be very useful.

Chromogranin A (CroA) and neuron specific enolase (NSE) are the most widely accepted serologic biomarkers of neuroendocrine tumors. CroA, a hydrophilic glycoprotein present in large dense core vesicles of neuroendocrine cells, has been identified as the most useful NET-related circulating marker. A major drawback of CroA is the lack of standardization of its assays, with several commercial kits, and different methodologies that hampers comparison among them[14]. Additionally, CroA levels are influenced by patient medication such as proton pomp inhibitors and medical conditions, such as chronic atrophic gastritis and chronic renal failure, presenting limitations for diagnosis in clinical practice[15,16]. NSE is a highly specific marker for neurons, peripheral neuroendocrine tissue, and APUD (Amine Precursor Uptake and Decarboxylation) cells, and can therefore serve as a biochemical marker for tumors derived from these cells[17]. Both CroA and NSE are general neuroendocrine markers.
We hypothesized that the PCF of cPanNETs has increased levels of CroA and NSE, and that this might assist in the differential diagnosis of cPanNETs. The aim of our study was to evaluate the accuracy of CroA and NSE in PCF for diagnosis of cPanNETs.

MATERIAL AND METHODS

Sample acquisition and case selection

The present preliminary longitudinal cohort study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1224).

PCF samples were selected from the EUS-FNA registry (of our Hospital), which started in 2008 and is used for diagnosis and clinical management of patients with pancreatic cysts. Clinical data, including cyst characteristics, PCF analysis, and follow-up are prospectively collected and registered in this database. All patients gave informed consent for EUS-FNA, standard PCF analysis, and remnant volume storage. After collection, PCF is centrifuged for 10 minutes at 2000g, for cytospin preparation for cytological analysis. The supernatant fluid is separated into two samples, the first 0.5 mL is sent for standard CEA and amylase evaluation and the remaining PCF is put on ice and stored at -80˚C no more

than 30 minutes after collection. The amount of PCF stored per patient varied according to the residual amount remaining after standard evaluation.

From 266 patients undergoing EUS-FNA for pancreatic cyst evaluation between 2008 and 2014, 102 frozen PCF samples were stored at -80˚C. We previously analyzed 52 frozen samples for *KRAS* and *GNAs* mutational analysis[18] and 82 for glucose (accepted for publication). The main criterion in selecting the cohort of patients for the present study, was to include all cPanNETs and for the remaining cysts, having more than 1 mL (4 aliquots of 0.25 mL) of frozen PCF available and a definitive diagnosis established, either surgical (pathology specimen) or clinicopathological (conclusive diagnosis with EUS-FNA cytology and prolonged surveillance, >24 months, with imaging stability). For the purpose of this study we divided the PCF samples into four main groups (see below), according to final cyst diagnosis.

We used 0.25 mL of frozen PCF to measure glucose (glucometer), CroA (CGA-RIACT®, Cisbio Bioassays, France), and NSE (LIAISON®XL NSE, DiaSorin, Italy) in all 16 samples.

In all patients, the symptoms and the EUS morphologic aspects of the cyst (location, size, thickened wall/septa or mural nodule, communication or dilation of the Wirsung, and peri-pancreatic adenopathies) had been registered, as well as PCF standard evaluation. Cytopathologic evaluation had been performed by experienced cytopathologists.

Standard PCF analysis with CEA, amylase, and cytology

CEA (Architect, Abbott; chemiluminescent immunoassay) and amylase (Architect, Abbott; kinetic colorimetric method) had been measured in all samples. A CEA level greater than 192 ng/mL prompted a classification of a mucinous cyst and lower than 192 ng/mL of a non-mucinous cyst. Cytological analysis of PCF, or surgical pathology specimens when available, classified the cysts into one of four groups: Group 1- cPanNETs; Group 2- Benign/inflammatory cysts (including SCAs and pseudocysts); Group 3- Pre-malignant/mucinous cysts (including IPMNs and MCNs with low-grade atypia (LG)); and Group 4- Malignant cysts (including an acinar cell tumor and an IPMN with high-grade atypia (HG)). After EUS-FNA procedure, patients were evaluated in pancreas clinic and referred for surgery (surgical cohort), imaging follow-up, or endoscopic drainage (non-surgical cohort).

Glucose, CroA, and NSE assays

All samples were blinded to the investigator. For glucose assay, 2 microliters of PCF were pipetted onto the side of the testing strip and analyzed using a Verio One Touch IQ glucometer (LifeScan Europe, Switzerland). The test range in the glucometer is 20-600 mg/dL, with a required sample volume of only 0.4 microliters. For numerical analysis, we considered a glucose measurement <20 mg/dL as 19 mg/dL.

CroA was measured in 0.25 mL of PCF sample by CGA-RIACT (Cisbio Bioassays, France), a solid-phase two site immunoradiometric assay, employing two monoclonal antibodies directed against sterically remote sites on the CroA molecule. The measuring range for CroA is 43 to 1100 ng/mL; sensitivity of the assay is 1.5 ng/mL; intra and inter-assay coefficients of variation, at a concentration of 144 ng/mL, are 3.8% and 5.7%, respectively; serum reference interval for CroA is <100 ng/mL. For numerical analysis we considered a CroA measurement <43 ng/mL as 42 ng/mL.

Determination of NSE was performed in a 1/20 dilution of the remaining PCF sample, due to low volume. We used the LIAISON®XL NSE (DiaSorin S.p.A, Saluggia, Italy), a chemiluminescence

immunoassay with two monoclonal antibodies. The measuring range is 0.04 to 200 ng/mL; sensitivity of the assay is 0.04ng/mL; intra and inter-assay coefficients of variation, at a concentration of 15 ng/mL, are 1.7% and 3.7%, respectively; serum reference interval for NSE is <18.3 ng/mL. For numerical analysis we considered an NSE measurement <0.8 ng/mL as 0 ng/mL.

Statistical Analysis

Descriptive analysis of quantitative data is expressed as mean ± SD or median and interquartile range (IQR). Fisher's exact test was used to assess differences between cPanNETs and other cyst types for dichotomous variables. We used the non-parametric Mann-Whitney test to compare CroA and NSE levels in two groups of cysts (cPanNETs *versus* other cyst types) and Kruskal-Wallis to compare variables in four groups of cystic lesions (cPanNETs, benign, pre-malignant, and malignant cysts). All tests were two-sided and statistical significance was defined as a p-value <0.05.

Receiver operator curves were generated and area under the curve (AUC) was calculated to evaluate the performance of CroA and NSE for classifying the cysts as either cPanNETs or non-cPanNETs. Correlation between CroA and NSE was measured using Spearman's rank correlation coefficient. The statistical analysis of the study was performed by Luisa Pereira, a biomedical statistician using SPSS Statistics version 24 (Armonk, NY).

RESULTS

Patients and pancreatic cysts characteristics

Sixteen patients were included in the study, including 9 females, with a mean age of 58±12 years (42-79). PCLs were mainly located in the head (7), followed by body (5) and tail (4) of the pancreas, with a mean size of 29.3±12.3 mm (15-60), and 31% (5/16) of cysts being larger than 3 cm. There were 4 cPanNETs, 7 benign (4 SCAs and 3 pseudocysts), 3 pre-malignant (3 IPMNs-LG), and 2 malignant cysts (1 acinar cell tumor and 1 IPMN-HG).

Gender, age, and cyst location did not differ between cPanNETs and other cyst types, or in EUS imaging features, including size, presence of septa, thickened wall, nodule, or peri-pancreatic adenopathy (Table 1). Concerning PCF analysis, CEA, amylase and glucose levels did not differ between cPanNETS and other cyst types, in contrast to CroA and NSE, which were significantly higher in cPanNETs (Table 1).

Table 1. Demographic and cyst characteristics in cPanNETs vs. non-cPanNETs			
	cPanNETs (n=4)	**Non-cPanNETs Cyst (n=12)**	***p* value**
Female (n, %)	3, 75%	6, 50%	0.392
Mean age±SD (range)	48.5±5.2 (43-55)	61.2±12.4 (42-79)	0.072
Cyst location (head/body/tail)	1/3/0	6/2/4	0.081
Cyst size(mm) mean±SD (range)	18.8±5.6 (15-27)	32.3±11.9 (20-60)	0.422
Septum or cyst wall thickening (n, %)	2, 50%	8, 67%	0.604
Nodules (n, %)	2, 50%	3, 25%	0.647
Peri-pancreatic adenopathies (n, %)	0, 0%	0, 0%	.
Acellular cytological exam (n, %)	2, 50%	2, 17%*	0.099
CEA (ng/mL) median (IQR)	23.5 (10.3-76.5)	51.5 (4.9-243)	0.716
Amylase (U/L) median (IQR)	129.5 (67-917.3)	6882.5 (61-27763.8)	0.332
Glucose (mg/dL) median (IQR)	96.0 (93.8-184.5)	54.0 (19-106.8)	0.142
CroA (ng/mL) median (IQR)	319.2 (199.4-529.2)	42.0 (42.0-110.4)	**0.005**
NSE (ng/mL) median (IQR))	412.7 (202.7-1297.7)	0 (0-14.6)	**0.002**
IQR, interquartile range; SD, standard deviation; CroA, Chromogranin A; NSE, neuron-specific enolase. For numerical analysis, we considered CroA measurement <43 ng/mL as 42 ng/mL and NSE <0.8 ng/L as 0 ng/mL. *Result is biased because the criteria to select other cyst types was having a diagnostic cytology and/or a surgical pathology diagnosis.			

Cro A and NSE assay results

CroA and NSE results obtained in each of the four diagnostic groups are presented in Table 2.

Table 2. Individual levels of CroA and NSE by cyst type with type of diagnosis.						
Groups of Cysts (n)	cPanNETs (4)	Benign Cysts (7)		Pre-malignant Cysts (3)	Malignant Cysts (2)	
Cyst Types (n)	cPanNETs (4)	SCA (4)	Pseudocysts (3)	IPMN-LG (3)	Acinar cell tumor (1)	IPMN-HG (1)
CroA (ng/mL)	160*, 318*, 320*, 683*	<43*, 133, 138, 334	<43, <43, <43	<43*, <43, <43	<43*	<43*
NSE (ng/mL)	1579*, 372*, 146*, 454*	<0.8*, 14, 33, <0.8	<0.8, <0.8, <0.8	<0.8*, <0.8, 51	15*	<0.8*
Surgical diagnosis (n)	4	1	0	1	1	1
Clinicopathological Diagnosis (n)	0	3	3	2	0	0
*Surgical pathology diagnosis						

Levels of CroA and NSE were significantly higher in cPanNETs (median values of 319.2 ng/mL and 412.7 ng/mL, respectively) than in non-cPanNETs (median values of <43 ng/mL and <0.8 ng/mL, respectively) (p=0.005 and p=0.002, respectively) (Figure 1).

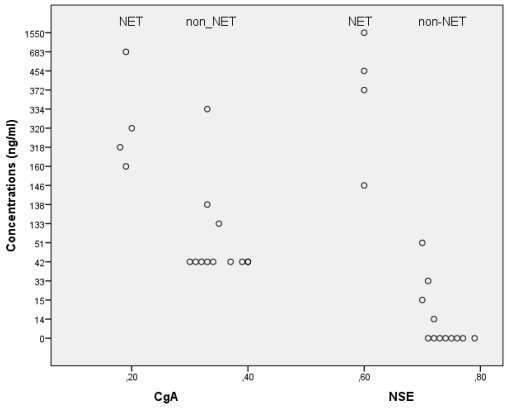

Figure 1. Comparison of cyst fluid Chromogranin A and NSE levels in patients with cPanNETs and non-cPanNETs

The ROC curve analysis showed an AUC=0.938 (95%CI 0.81-1) for CroA and an AUC=1 (95%CI 1-1) for NSE, with optimal cut-off values for the diagnosis of cystic pNETs >148.8 ng/mL for CroA and >98.5 ng/mL for NSE (Table 3).

Table 3. ROC curve analysis of CroA and NSE in PCF for diagnosis of cPanNETs (n=4 vs. n=12)

	AUC	95%CI	p-value	Optimal cut-off	Sensitivity (%)	Specificity (%)
CroA (ng/mL)	0.938	(0.81-1)	0.011	148.75	100	92
NSE (ng/mL)	1	(1-1)	0.004	98.5	100	100

ROC, receiving operating characteristics; CroA, Chromogranin A, NSE, neuron specific enolase; PCP, pancreatic cystic fluid; cPanNETs, cystic pancreatic neuroendocrine tumors

Correlation of CroA and NSE assays

There was a strong positive correlation between CroA and NSE (r_s=0.644, p=0.007). Thus, increased levels of CroA are associated with increased NSE level.

DISCUSSION

In this preliminary study we found that measuring CroA and NSE in PCF was highly specific for diagnosing cPanNETs, that were otherwise identical to other PCLs. CroA alone allowed the differentiation of cPanNETs using the cut-off value of >149 ng/mL, as did NSE using the cut-off value of >99 ng/mL, with excellent sensitivities of 100% and 100% and specificities of 92% and 100%, for CroA and NSE respectively.

In our study no clinical or imaging features could differentiate cPanNETs, except a tendency for younger age, with a mean age of 49 years old in cPanNETs patients *versus* 61 in non-cPanNETs (p=0.072), compared to a median age of 58 years old in cPanNETs in a previous series[9], and a predominant location in the pancreatic body in 3/4 cysts (p=0.08), similar to results in the same series[9]. Also in the same study, pNETs were rarely suspected through the use of cross-sectional imaging, with approximately 40% of lesions showing neither cyst wall thickening nor nodularity[9]. In our series only 2/4 (50%) of cysts showed these features. This imaging dilemma makes cytology a cornerstone in cPanNETs diagnosis and targeting the cyst wall during FNA is recommended. In our study 50% (2/4) of cPanNETS FNAs were acellular, compared to 21% (4/19) of non-diagnostic cytologies in a study by Yoon *et al* [19] and 21% (5/24) in a study by Ho *et al* [9] This may be explained by our reduced number of cPanNETs. Standard PCF biochemical analysis in cPanNETs shows low CEA (<192 ng/mL) and high glucose (>50 mg/dL), which are non-diagnostic and identical to other more common non-mucinous cysts, such as SCAs and pseudocysts. This common biochemical profile and a non-diagnostic cytology may erroneously suggest a benign cyst, whereas in fact we are dealing with a potentially malignant cyst.

This panorama emphasizes the need of biomarkers which help in further characterizing PCLs, namely cPanNETs. CroA and NSE, due to their role as tumor markers in the serum of both functioning and non-functioning NETs [20], are possible candidates. These have been evaluated in two previous studies. The first was negative for CroA, but included only two cPanNETs and used a different assay (DIAsource Immuno Assays, Louvain-la-Neuve, Belgium)[21]. The second included five cPanNETs and was positive for both CroA and NSE, but used different assays, with a Kryptor system (BRAHMS GmbH, Thermo Scientific) for CroA and a Cobas E analyzer (Roche Diagnosis) for NSE[22].

Our study included four cPanNETs and both CroA and NSE yielded excellent discrimination with other PCLs in contrast to conventional biochemical biomarkers (CEA, glucose, and amylase)

and/or cytology. As CroA and NSE showed a good correlation between them, either seems to be adequate for cPanNET diagnosis. In our study we used the CroA (CGA-RIACT®, Cisbio Bioassays, France) and the NSE (LIAISON®XL NSE, DiaSorin, Italy) assays. The discrepancy with previous published studies may be due to different assays, which, as described for serum samples, may preclude the comparison of results[14].

There are several limitations in our study. First, the small number cysts, including cPanNETs analyzed, with possible selection bias, because only lesions with conclusive FNAs were selected for comparison. Also, only half (8/16) of the cysts had a surgical pathology diagnosis; the other half had a clinicopathological diagnosis. This may have precluded a correct diagnosis of some lesions on the one hand but reduced the selection bias associated with a mandatory surgical pathology diagnosis on the other, that would have limited the study sample to lesions with imaging or cytological high-risk features. Besides, when comparing surgical pathology and clinicopathological diagnoses, CroA and NSE levels were not different (Table 2). Another limitation was the reduced number of PCLs included in the study, which did not allow the determination of CroA and NSE levels in other rare PCLs, such as lymphangioma or solid-pseudopapillary neoplasm, among others. Finally, it would also be interesting to measure serum levels of CroA and NSE and to seek correlation with PCF levels.

In conclusion, our preliminary results suggest that elevated levels of CroA and NSE in PCF accurately distinguish cPanNETs from other cyst types. Determination of CroA and NSE levels may complement a non-diagnostic cytology result, especially in cases in which low CEA and/or high glucose levels may erroneously suggest a benign cyst. More extensive studies in other centers, if possible, using the same assays for diagnosis, may validate our findings and lead to a uniform interpretation of these promising test results.

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3.4. Methylation changes at the *GNAS* imprinted locus in pancreatic cystic neoplasms are important for the diagnosis of malignant cysts. Submitted for publication.

Abstract

Background: *GNAS* mutations are characteristic of intraductal papillary mucinous neoplasms (IPMNs). Pancreatic ductal adenocarcinomas (PDACs) harbouring *GNAS* mutations originate in IPMNs. *GNAS* is a complex imprinted *locus* that produces 5 transcripts regulated by differential methylated regions (DMRs), *NESP55*, *GNASAS*, *GNASXL*, *GNAS1A* and *GNAS*. In this study, we evaluated if methylation changes in the DMRs of GNAS *locus* contributed to malignant progression of pancreatic cysts (PCs).

Methods: *GNAS locus* methylation was analysed in archival pancreatic cyst fluid (PCF), obtained by Endoscopic Ultrasound with fine-needle aspiration (EUS-FNA), by *methylation specific-multiplex ligation dependent probe amplification* (MLPA). Results were normalized and analyzed using Coffalyser.Net software.

Results: Fifty-two PCF samples obtained by EUS-FNA and previously characterized for *KRAS* and *GNAS* mutations, were studied. The final diagnoses were surgical (11) and clinicopathological (41), including 30 benign cysts, 14 pre-malignant cyst, and 8 malignant cysts. Methylation changes at *NESP55*, *GNASAS*, *GNAS1A*, and especially *GNASXL* were more frequent in malignant cysts and were useful for their diagnosis. A combined variable defined as "*GNAS locus* methylation changes" was significantly associated with malignancy (6/8 malignant cysts and only 2/20 benign cysts) and improved classification. Hypermethylation in both maternally (*NESP55*) and paternally (*GNASXL*) derived promoters was found in 3/3 PDACs.

Conclusion: This is the first study to identify methylation changes in the *GNAS locus* improving the diagnosis of malignant PCs and suggesting a role in progression to PDAC.

Keywords: IPMN; Pancreas Cyst; Methylation; Biomarker; *GNAS* locus; Pancreatic Neoplasm

Core Tip: Pancreatic cystic lesions are a clinical dilemma due to risk of malignancy. Somatic mutations of *GNAS* are characteristic of IPMNs.

We found methylation changes in differential methylated regions (DMRs) at the *GNAS locus* in pancreatic cyst fluid predominantly of malignant cysts. Methylation changes in *GNAS locus* may improve the diagnosis of malignant cysts and shed light to the development of novel therapeutic approaches for pancreatic cancer.

Main Text:

Introduction

Pancreatic cystic lesions (PCLs) constitute a clinical dilemma due to indeterminate risk of malignancy, including benign cysts (BCs), pre-malignant cysts (PMCs), and malignant cysts (MCs) [1]. Intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) are cystic precursors of pancreatic ductal adenocarcinoma (PDAC), allowing early diagnosis. [2]

Somatic mutations in *GNAS* are characteristic of IPMNs [3,4], but their role in carcinogenesis is unclear, with early occurrence precluding prediction of dysplasia [5,6]. However, if detected in PDACs, somatic mutations in *GNAS* are specific for an IPMN origin [3].

GNAS is a complex imprinted *locus* in the long arm of chromosome 20 (20q13.32) [7] that encodes the α-subunit of the stimulatory heterotrimeric G protein (Gsα), a ubiquitous signaling protein, translated from *GNAS* exons 1-13. This *locus* encodes four monoallelic (*NESP55, AS, XL, 1A)* and one biallelic (*Gs*α) transcripts, due to differentially methylated regions (DMRs) in paternal and maternal alleles, denominated imprinting [8,9]. Paternal methylation of *NESP55* and maternal methylation of *AS, XL* and *1A* lead, respectively, to maternal and paternal allele expressions, with Gsα biallelically expressed in most tissues, due to absent methylation [10].

Epigenetic alterations in the *GNAS locus* have not been previously evaluated in PCLs. Methylation of DMRs may occur at the somatic level and modulate Gsα expression, [10,11] leading us to hypothesize that methylation changes in DMRs at the *GNAS locus* could contribute to tumor progression of PCLs. To test our hypothesis, we performed a longitudinal cohort pilot study of PCLs and analyzed *GNAS locus* methylation in pancreatic cyst fluid (PCF) samples.

Methods

Case Selection

All patients gave informed consent and the study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1143).

For this study we performed molecular analysis in samples of 52 patients with more than 1 mL of PCF stored in the biorepository of our hospital, with sample processing and storage described in a previous publication [12]. Clinical data, including demographics, cyst characteristics, and treatment decision have been prospectively registered.

After undergoing EUS-FNA, patients were evaluated in clinics, and referred for surgery (surgical cohort, surgical pathology diagnosis) or imaging surveillance, palliation, or endoscopic drainage (clinical cohort, clinico-cytological diagnosis), when surgery was not clinically indicated and a surgical pathology specimen was not available for diagnosis. The diagnostic criteria for the clinical cohort were determined a priori by one of the investigators (SF) after reviewing imaging features, PCF levels of CEA and cytology analysis of PCLs, all with a prolonged imaging and clinical follow up (at least of 24 months). To evaluate *GNAS locus* methylation distribution and the performance of methylation analysis for cyst diagnosis, PCLs were further classified into one of three groups: Group 1) Benign cysts (BCs), including neoplastic benign and inflammatory cysts (serous cystadenomas (SCAs), pseudocysts, and lymphangiomas); Group 2) Mucinous pre-malignant cysts (PMCs), including IPMNs and MCNs with low grade atypia (LG); Group 3) High-risk/malignant cysts (MCs), including cystic PDACs, IPMNs with adenocarcinoma (ADC) or high grade atypia (HG), MCN-HG, and neuroendocrine cystic tumors (NETs).

Patients and specimens

The samples studied were predominantly from female patients (35/52, 67%) with a mean age of 59±15 years (29-91), and 22 PCLs were in the head, 20 in the body, 9 in the tail, with one case of multiple pancreatic locations. The mean cyst size was 3.9±2.3cm (1-10), CEA level in PCF was >192ng/ml in 17/52 (33%), and malignant/atypical cytology was present in 11/52 (21%) PCF samples, as presented in Table 1.

Table 1. Demographics and clinical characteristics of the study population.

Female gender, n (%) (n=52)	**35 (67,3%)**
Mean age at EUS-FNA, y, mean ± SD (interval)	**59,1 ± 14,8 (29-91)**
Cyst location, n (%) (n=52)	
Head	**22 (42,3%)**
Body	**20 (38,5%)**
Tail	**9 (17,3%)**
Multiple cyst locations	**1 (1,9%)**
Cyst size, cm, mean ± SD (interval)	**3,9 ± 2,3 (1-10)**
Cyst size >3 cm, n (%)	**29 (55,8%)**
Cyst with nodule/mass, n (%)	**18 (34,6%)**
EUS Imaging, n (%) (n=52)[13]	
No high risk features	**13 (25%)**
1 high risk feature	**29 (55,8%)**
≥2 risk features	**10 (19,2%)**
PCF CEA, n (%) (n= 52)	
CEA< 192ng/mL, n (%)	**31 (59,6%)**
CEA≥ 192ng/mL, n (%)	**17 (32,7%)**
No result available	**4 (7,7%)**
PCF cytology, n (%) (n=52)	
Non-diagnostic	**27 (51,9%)**
Negative for malignancy	**14 (26,9%)**
Suspicious/malignant	**10 (19,2%)**
NET	**1 (2%)**
Treatment decision, n (%) (=52)	
Follow up	**34 (65,4%)**
Surgery	**11 (21,2%)**
Endoscopic drainage	**1 (1,9%)**
Palliation (symptomatic or chemotherapy)	**6 (11,5%)**

EUS-FNA, Endoscopic ultrasound with fine needle aspiration; High-risk features: cyst size ≥3 and solid component or thick wall or dilated Wirsung (>10 mm)[13]; CEA, carcinoembryonic antigen; NET, neuroendocrine tumor; PCF, pancreatic cyst fluid; SD, standard deviation

These fifty-two PCF samples obtained by Endoscopic Ultrasound with fine-needle aspiration (EUS-FNA) have been previously characterized for *KRAS* and *GNAS* mutations [[12]], that were present in 9 and 2 samples, respectively.

The final diagnoses, 11 surgical and 41 clinicopathological, encompassed 30 BCs (SCAs, pseudocysts, and lymphangiomas), 14 PMCs (IPMNs and MCNs), and 8 MCs (1 cystic PDAC, 1 IPMN-ADC, 1 NET, and 5 Mucinous-malignant).

Methylation Analysis and Categorization

For this study, DNA was extracted from 0.250 ml of archival PCF. Methylation analysis of the *GNAS locus* was performed by *methylation specific-multiplex ligation dependent probe amplification* (MS-MLPA)

(SALSA MS-MLPA ME031-B1, MRC-Holland®), according with the manufacturer. MS-MLPA fragments were analyzed on the Applied Biosystems® 3130 Genetic Analyzer (ThermoFisher Scientific) using the GeneMapper® software. Results were normalized and analyzed using Coffalyser.Net software (MRC-Holland®).

We studied methylation in 4 DMRs, *NESP55, GNASAS, GNASXL, GNAS1A* and in the biallelic expressed *Gs*α, including two exonic regions. DMRs were classified as hypermethylated or hypomethylated, according to the percentage of methylation obtained using the Coffalyser.net software recommended by the manufacturer, if methylation percentage was, respectively, above or below the reference values plus or minus twice the standard deviation. The normal methylation of *NESP55* is approximately 50%, as only the paternal allele is methylated, similarly to the percentage of methylation in *GNASXL*, *GNASAS,* and *GNAS1A*, as only the maternal alleles are expected to be methylated. The methylation of *Gs*α exon 1 is usually absent, as neither maternal nor paternal alleles are methylated. Methylation of *Gs*α exonic regions (exons 9 and 13) is usually near 100%, as both maternal and paternal alleles are methylated. The MS-MLPA kit comprised three methylation sensitive probes for *NESP55,* three for *GNASAS*, five for *GNASXL*, two for *GNAS1A,* and four for *Gs*α methylation evaluation.

Statistical Analysis

The methylation levels obtained for each of the individual DMRs and for each individual MS-MLPA probe were calculated and converted into a categorical variable defined as: 1) hypomethylated if methylation level obtained was below the cut-off level minus twice the standard deviation (SD); 2) hypermethylated if the methylation level obtained was above the cut-off level plus twice the SD; and 3) normally methylated if neither criteria 1) or 2) were met. A combined variable, including hypermethylation at upstream DMRs or intragenic hypomethylation of *GNAS locus,* defining "*GNAS locus* methylation changes" pattern was created. For (epi)genotype-phenotype associations, Fisher's exact test and chi-square test were performed. Methylation analysis in mucinous and malignant cysts was also represented by boxplot and Mann-Whitney was used to assess the difference of median methylation values. The diagnostic accuracy of PCF biomarkers was assessed by receiver operating characteristics (ROC) curve analysis. Statistics were performed using SPSS Statistical software, version 23 (Armonk, NY), with a p value <0.05 considered as statistically significant.

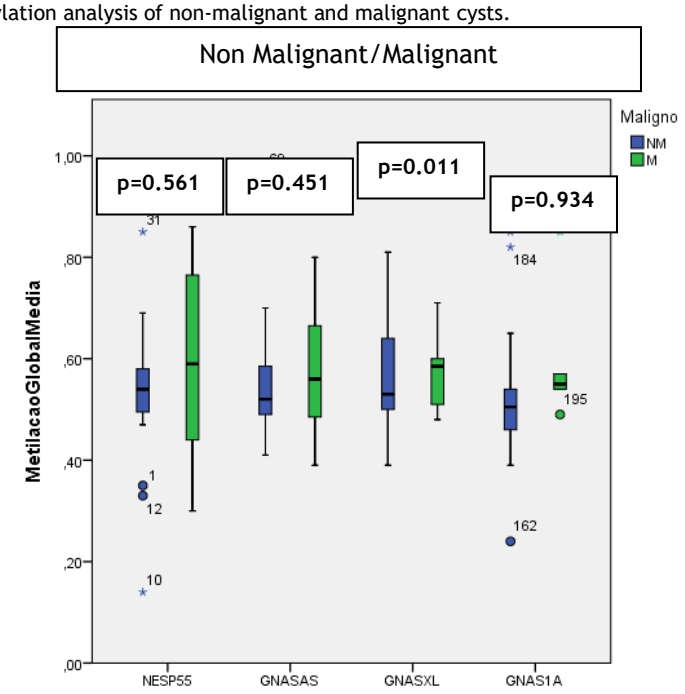
RESULTS

GNAS locus methylation was informative in 38/52 (73%) PCF samples, with the remaining (14/52) non-informative due to inadequate quality/quantity of DNA, and rarely, to copy-number variation (probe ratios below 0.7 or above 1.3, regarded as indicative of heterozygous deletion or duplication, respectively, according with the manufacturer (Coffalyser.Net software, MRC-Holland®). Methylation changes at *NESP55*, *GNASAS*, *GNAS1A,* and especially *GNASXL* were more frequent in MCs (Table 2), presenting wider methylation levels of these DMRs compared to non-malignant cysts, which showed methylation levels around 50% in imprinted alleles (Figure 1).

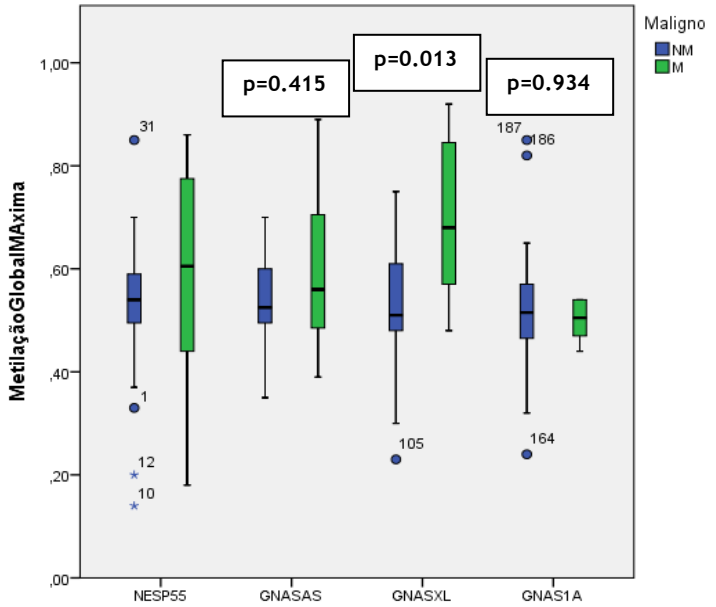
Table 2. Frequency of *GNAS locus* methylation changes in malignant, mucinous and benign cysts				
Informative cyst fluid methylation analysis (38 samples)	**Malignant (n=8)**	**Mucinous pre-malignant (n=10)**	**Benign (n=20)**	***P*-value**
***NESP55* hypermethylation, n (%)**	3 (37.5%)	0 (0.0 %)	1 (5.0%)	0.053
***GNASAS* hypermethylation, n (%)**	3 (37.5%)	1 (10.0%)	3 (15.0%)	0.065
***GNASXL* hypermethylation, n (%)**	4 (50%)	0 (0.0%)	2 (10.0%)	0.004
***GNAS1A* hypermethylation, n (%)**	1 (12.5%)	0 (0.0%)	0 (0%)	0.0355
***GNAS locus* methylation changes, n (%)**	6 (75.0%)	0 (0.0%)	2 (6.7%)	0.000
GNAS locus methylation changes, DMR hypermethylation or *GNAS* intragenic hypomethylation				

GNAS locus methylation changes, DMR hypermethylation or *GNAS* intragenic hypomethylation.

Figure 1. Methylation analysis of non-malignant and malignant cysts.



Mean value of the percentages of methylation obtained in all probes.



Single probe with the highest percentage of methylation.

Based on the influence of methylation changes at DMRs in the modulation of *GNAS* transcription[10,11] and on the suggested role for hypomethylated exons in transcription regulation and its overlap with predicted enhancers[13] we defined a combined variable documenting "*GNAS locus* methylation changes": 1) presence of hypermethylation in at least two DMRs or in one DMR for all MLPA probes or 2) presence of intragenic hypomethylation of *GNAS* in at least two exonic regions. Notably, "*GNAS locus* methylation changes" was significantly associated with malignancy (6/8 MCs and only 2/20 BCs) (Table 2) and it is of note that one of these two BCs was later diagnosed as pancreatic cancer. Moreover, the "*GNAS locus* methylation changes" variable improved MCs classification in samples with clinicopathological diagnosis (possible diagnostic uncertainty) as well as surgical diagnosis (definitive diagnosis but limited number of cases), further supporting our results.

Interestingly, simultaneous hypermethylation in *NESP55 and GNASXL* DMRs was detected exclusively in 3/3 PDACs. Hypomethylation in two exonic *GNAS* regions (exons 9 and 13) was detected in the only NET in this series.

Additionally, "*GNAS locus* methylation changes" was associated with symptoms, *KRAS*/*GNAS* mutations, and malignant/atypical cytology, but not with patient gender, age, or CEA level in PCF (Table 3), with the AUC analysis revealing better performance for diagnosis of MCs than cytology (Table 4).

Table 3. Frequencies of distinct clinical features and pancreatic cystic fluid analysis in the two groups, with or without *GNAS locus* methylation changes.

Cyst fluid samples	*GNAS locus* methylation changes	No *GNAS locus* methylation changes	*P*-value
Female	63%	75%	0.486
Age >65 years old	50%	40%	0.216
Symptoms	63%	17%	0.008
CEA >192 ng/mL	63%	25%	0.133
KRAS/GNAS mutation	63%	11%	0.008
Cytology (malignant/atypical)	63%	7%	0.003

GNAS locus methylation changes, DMR hypermethylation or *GNAS* intragenic hypomethylation.

Table 4. Area Under the Curve for diagnosis of mucinous and malignant cysts.

	Mucinous cysts				Malignant cysts			
Variables	**AUC**	**P-value**	**Confidence Interval**		**AUC**	**P-value**	**Confidence Interval**	
			Lower Limit	**Upper Limit**			**Lower Limit**	**Upper Limit**
CEA (mg/dL)	.889	.002	.720	1.000	.812	.038	.579	1.000
Cytology	.598	.443	.349	.847	.771	.072	.571	.970
Mutation (*KRAS*/*GNAS*)	.833	.009	.634	1.000	.841	.023	.615	1.000
Met_*NESP55*	.620	.35	.370	.869	.759	.085	.481	1.000
Met_*AS*	.590	.483	.339	.841	.741	.108	.461	1.000
Met_*XL*	.474	.841	.228	.721	.629	.389	.357	.902
Met_*1A*	.513	.92	.262	.764	.565	.667	.261	.868
GNAS_locus methylation changes	.645	.256	.400	.891	.971	.002	.901	1.000

CEA, carcinoembryonic antigen; Met, methylation changes; AUC, area under the curve

Discussion

Aberrant DNA methylation in PCF of IPMNs progressing to high-grade dysplasia and carcinoma has been described [14], but *GNAS locus* methylation was not studied therein.

We report for the first-time methylation changes in the *GNAS locus,* namely hypermethylation of *GNASXL*, *NESP55*, *GNASAS* and *GNAS1A* in PCLs. Notably, hypermethylation of *GNASXL*, and especially the combined variable "*GNAS locus* methylation changes", were associated with malignancy, suggesting potential to be used for diagnosis of MCs and for monitoring cancer progression, if confirmed in larger series. Indeed, hypermethylation of *GNASXL* has been associated to *GNAS locus* gain of function [10] and although its possible association with malignant progression remains poorly understood, *GNAS* oncogenic potential appears to be unquestionable [3-5,10,15]. Moreover, somatic DNA methylation has been shown to drive transcription within the imprinted *Gnas* cluster [11], further supporting our results. *NESP55* appears also to regulate imprinting at the *GNAS* complex *locus*, and its hypermethylation in the maternal allele may lead, similarly to maternal deletion, as previously described, to subsequent modulation of *GNAS* [10].

Herein, the detection of hypermethylation in both maternally (*NESP55*) and paternally (*GNASXL*) derived promoters, and therefore global increase of methylation in these two DMRs, detected exclusively in PDAC, further suggests a role of *GNAS* in malignant progression of PCL. Interestingly, the detection of exonic *GNAS* hypomethylation in the pancreatic NET is in agreement with the recent findings showing that pancreatic NETs are genetically and phenotypically related to pancreatic ductal adenocarcinoma, having a closer relationship to ductal adenocarcinomas than to neuroendocrine tumors G3 [16]. In agreement with the role of *GNAS* in the progression to PDAC is also the recent finding that overexpression of mutant *Gnas*, resulting in constitutive activation of *Gsa*, in a mouse model of *Kras*^{G12D}-driven pancreatic cancer led to the formation of moderately differentiated PDAC that were locally invasive and increased MAPK activation [17] .

Although copy-number alterations, which could in part explain some of the methylation changes found, were detected in only one case, we cannot exclude the presence of uniparental disomy (UPD) associated copy-neutral loss of heterozigoty (LOH), as previously described by Bastepe to explain *GNAS* methylation changes [18]. An analysis of LOH in the *GNAS locus* would be needed to evaluate uniparental disomy (UPD) associated copy-neutral LOH (which can often be segmental) and investigate if some of these methylation alterations may indeed reflect epigenetic alterations, or could instead be explained by acquired UPD (at least in part). Nevertheless, independently of their cause (epigenetic or acquired UPD), the resulting methylation alterations detected in the *GNAS locus* DMRs appear to be related to malignant progression and may improve MCs diagnosis. Our study may contribute to propel the current epigenetic landscape of PCs, similarly to recent studies documenting a role for methylation markers in discriminating pancreatic neoplasia [19,20], possibly offering an opportunity for early diagnosis for pancreatic cancer.

Ultimately, the significant association of *GNAS locus* methylation changes to malignant behavior suggests a role for modulation of *GNAS* expression in the malignant progression of PCs which may be relevant for the development of novel therapeutic approaches for pancreatic cancer. Due to small sample size and poor DNA yield, the final analysis was based on 8 samples with HGD/cancer. Although the small sample size and lack of validation in an independent sample set significant limits the present study, our pilot data may be the basis for exploring *GNAS* methylation in larger, well-characterized sets of samples that may represent future validation studies.

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Chapter V.

Discussion

The current increase in pancreatic cysts diagnosis requires an accurate pre-operative distinction between the far more frequent benign and low-risk cysts, which may even be released from surveillance programs, and mucinous lesions with malignant potential that require surveillance or even surgery, when evolution to HGD or early cancer occurs.

CEA is currently considered the best biomarker of mucinous cysts, although in clinical practice it is suboptimal, due to the significant amount of PCF required for analysis, with around one third of non-diagnostic samples due to insufficient PCF[124], and about one third of PCLs with a CEA level, between 5 and 192 ng/mL, considered indeterminate for diagnosis.[86] Cytology of PCF is the best marker of malignancy in clinical practice, but a large proportion of samples are non-informative due to scant cellularity.

More accurate diagnostic tools would lead to fewer unnecessary MRIs for surveillance without affecting early cancer detection and adequate surgical referral.

For the purpose of the present work, we conducted an evaluation of biomarkers to move the laboratory to the bedside.[36] The framework for discovery and validation of cancer biomarkers encompasses five distinct phases: preclinical exploratory (phase 1), clinical assay and validation (phase 2), longitudinal retrospective (phase 3), prospective screening (phase 4), and cancer control (phase 5). A successful performance in phase 3 is the minimum required for a biomarker validation in clinics.

The original studies presented in this work correspond to phase 3 (longitudinal retrospective). In order to validate these cystic fluid biomarkers for inclusion in daily clinical practice, we must first show that they perform better than the current standard – CEA and cytology.

Our samples were all collected by EUS-FNA in patients who might in the future (but not immediately) require surgical treatment but were kept under surveillance. Our goal was to assess these biomarkers' capacity to predict disease outcome, by retrospective assessment.

1. Current Evidence of *KRAS* Mutations for Pancreatic Cyst Diagnosis

The current standard diagnosis of mucinous cysts is EUS-FNA for CEA and cytology of PCF, although their diagnostic performance is considered suboptimal. The increasing number of asymptomatic PCLs diagnosed, most with potential for malignancy, justifies the growing need to find accurate and affordable tests for diagnosis, in order to reduce surgeries on benign cysts, and diagnose and resect early malignant lesions with favorable prognosis. The study of DNA molecular markers, particularly *KRAS* mutations[19,20,21,22,23,1,24,25,26], an early somatic event in pancreatic carcinogenesis, has been suggested to fulfill this need.

In a first meta-analysis (1.1.), we studied the diagnostic accuracy of *KRAS* mutations in malignant and significant cysts (malignant and pre-malignant cysts under surveillance) as compared to clinical routine diagnosis of CEA and cytology obtained by EUS-FNA. A total of 16

studies were included corresponding to 3429 patients, of which 731 (21%) had undergone surgical resection and had a surgical pathology specimen available for diagnosis. Within this population a precise diagnosis was assured.

The pooled sensitivity of *KRAS* mutations for malignant cyst diagnosis was 0.43 (95% CI, 0.34-0.53) and the pooled specificity was 0.62 (95% CI, 0.56-0.68), while for the larger group of significant cysts we found 0.46 (95% CI, 0.42-0.51) and 0.97 (95% CI, 0.92-0.99), respectively. The area under the sROC curve ± SE was 0.56 ± 0.07 in malignant cysts and 0.53 ± 0.14 in significant cysts. The results of the studies had greater variation for diagnosis of malignant cysts, as shown by the wide confidence region.

Cytology of PCF, the current standard for malignant cysts diagnosis, had a pooled sensitivity of 0.37 (95% CI, 0.27-0.48) and specificity of 0.96 (95% CI, 0.93-0.98) whereas for significant cysts it was 0.19 (95% CI, 0.13-0.25) and 0.94 (95% CI, 0.86-0.98), respectively. The area under the sROC curve ± SE was 0.78 ± 0.13 in malignant and 0.48 ± 0.15 in significant cysts.

Since only four studies (with few patients) allowed the evaluation of accuracy of CEA >192 for diagnosis of malignant cysts, and this biomarker is not considered useful for these cysts, we restricted the analysis of CEA to significant cysts. The pooled sensitivity was 0.58 (95% CI, 0.52-0.65) and a pooled specificity was 0.90 (95% CI, 0.76-0.97). The area under the sROC curve ± SE was 0.69 ± 0.12.

According to our results, *KRAS* mutation had a lower diagnostic accuracy than CEA and cytology, and for the present time, should not replace standard PCF analysis. Due to their early occurrence in pancreatic carcinogenesis, *KRAS* mutations have a significant rate of false-positive results if a malignant cyst diagnosis is under consideration.

This meta-analysis settled the intended use and clinical role of *KRAS* mutational analysis in the present time to be limited to patients with an undefined CEA level and a non-diagnostic cytology, serving only as a complementary diagnostic test due to its limited accuracy. NGS was used in all the studies evaluated, and its advantages include a very high sensitivity for detection of genetic mutations, using pre-defined panels of cancer genes, and even samples with limited DNA content, such as PCF, can be appraised. Nevertheless, disadvantages of NGS include storage, infrastructure, data processing, trained personnel, and large numbers of samples required to become cost-effective, making its widespread use in clinical practice difficult to achieve. Moreover, large multicenter validation studies and additional trials confirming its clinical relevance in patients' outcomes are also needed, including early cancer diagnosis, number of surgeries of benign lesions avoided, and prognostic value in cysts requiring periodic surveillance.

In a second meta-analysis (1.2.), we compared the diagnostic accuracy of molecular analysis with microforceps biopsy (MFB) of the cystic wall for diagnosis of PCLs in a cohort of surgical patients - a cohort in which diagnostic accuracy is guaranteed.

Both molecular analysis of PCF and MFB obtained by EUS-FNA are promising tools for diagnostic improvement of PCLs, and the comparative performance of both methods have not been studied.

The numerous studies showing that genetic analysis of aspirates obtained by EUS-FNA could provide a better characterization of PCLs than CEA and cytology[19,20,21,22,23,1,24,25,26] used NGS, with the above described drawbacks, and the clinical need of better diagnostic tests in PCLs led to the development of a through-the-needle miniature biopsy device for use during EUS-FNA.

The Moray micro forceps biopsy device (US Endoscopy, Mentor, Ohio) is disposable and can pass through the standard 19-gauge EUS-FNA needle that is already used routinely, allowing tissue sampling from the cyst wall, septa or mural nodules. The histological evaluation of the epithelial architecture and subepithelial stroma may potentially improve diagnosis. Several recent studies have investigated its accuracy.[125,45,126 ,46]

In the second meta-analysis, a total of eight studies, including 1 206 patients, of which 203 (17%) who were referred for surgery, and had a surgical pathology specimen were analyzed. The performance of molecular analysis and MFB were identical for diagnosis of benign cysts, while molecular analysis performed better for diagnosis of both low and high-risk mucinous cysts, with sensitivities of 0.89 (95%CI: 0.79-0.95) and 0.57 (95%CI: 0.42-0.71), specificities of 0.88 (95%CI: 0.75-0.95) and 0.88 (95%CI: 0.80-0.93) and AUC of 0.96 and 0.92, for molecular analysis and MFB respectively. The diagnostic yield was higher in MFB than in molecular analysis (0.73 vs 0.54, respectively), but the rates of correctly identified cysts were identical (0.73 with 95%CI: 0.62-0.82 vs 0.71 with 95%CI: 0.49-0.86, respectively).

This study underscores the diagnostic value of both tests, with higher diagnostic accuracy of molecular analysis for both low-risk and high-risk mucinous cysts.

In addition to significant costs, the technical complexity of the test makes the implementation of molecular analysis in clinical practice unlikely. On the other hand, MFB needs to be demonstrated in larger studies to be safe and allow tissue acquisition with the histological criteria needed for a correct diagnosis of PCLs. With the described results, we believe that for the present time, molecular analysis and MFB should be recommended only as complementary or second line tests in the event that CEA and cytology of PCF are non-diagnostic. If MFB proves to be safe and to improve the diagnosis of PCLs in larger studies, it may be quickly implemented in clinics. It may be particularly useful for benign lesions, for which neither surgery nor surveillance are required, with diagnosis uncertainty due to limitations of current diagnostic tests. However, both tests still require validation studies.

Finally, the successful massive implementation of these new diagnostic tools requires their recognition as universal, safe, highly accurate, and first line tests. Their validation as complementary tests in patients with non-diagnostic CEA/cytology are insufficient.

2. Original Research for Mucinous Cyst Diagnosis

Despite the results of our meta-analysis[32], some earlier studies showed that molecular analysis of aspirates obtained by EUS-FNA provided a better characterization of pancreatic cysts than standard CEA and cytology.[19,20,21,22,23,1,24,25,26]

Although NGS is a more recent and extremely sensitive technique that allows the study of entire panels of cancer genes that may bring genetic analysis into routine clinical practice, Sanger sequencing is still considered the standard diagnosis for molecular analysis.

In our first original study (2.1.), we evaluated the clinical impact of *KRAS*/*GNAS* mutation analysis in PCF for classification and decision-making of PCLs. Our cohort of patients presents predominantly benign and low-risk pancreatic cysts, representing the current burden of PCLs in clinical practice.

From 52 frozen samples of PCF obtained by EUS-FNA between 2008-14, additionally to PCF cytology and CEA, we studied *GNAS* (exons 8 and 9) and *KRAS* (exons 2 and 3) mutations using Sanger sequencing. Cysts were classified as mucinous in 21 (40%) patients (7 malignant, 14 low-risk) and non-mucinous in 31 (60%).

KRAS mutations were detected in 9 (17%) and *GNAS* in 2 (4%) PCF samples. Patients harboring cysts with *KRAS* mutations were older (p=0.01), cysts were more commonly mucinous (p=0.001) and with a malignant cytology (p=0.01). In the present series, *KRAS* mutations were present in both low-risk and malignant mucinous lesions. For identifying mucinous lesions, CEA >192 ng/mL performed better (AUC=0.93), whereas for malignant/high-risk mucinous lesions, EUS imaging had the best accuracy (AUC=0.88). After molecular testing, re-allocation in cyst classification occurred in 10 patients, but correctly in only 2. It allowed the diagnosis of more low-risk mucinous cysts (previously classified as inflammatory) and it did not confirm 1 high-risk cyst (that, in fact, presented a false-positive malignant cytology). The other 8 incorrect classifications with *KRAS* mutations, included 6/12 low-risk mucinous cysts that would be classified as “non-mucinous” (false-negative results in all cases) and 2/8 high-risk mucinous cysts that would be classified as “non-malignant” (false-negative results).

Our results did not support the added value of *KRAS* and/or *GNAS* mutations for the diagnosis of PCLs in comparison with conventional tests. Although *KRAS* mutation occurred predominantly in mucinous and malignant cysts, CEA level for low-risk mucinous cysts and combined imaging and cytology for high-risk mucinous/malignant cysts, were more accurate.

Two technical aspects must be pointed out. One refers to the lower sensitivity of Sanger sequencing compared to NGS. Also, the predominance of low-risk cyst types in our patient population could account for these results, in contrast with earlier studies in which NGS for molecular analysis and surgical series are predominant. On the other hand, our cohort better represents the most frequent cyst types in clinical practice, while in most publications

surgical series predominate, with the inherent selection bias favoring high-risk PCLs, which are much rarer in clinical practice.

The understanding of these apparent substandard results of genetic markers for pancreatic cyst diagnosis, in which *KRAS* and *GNAS* mutations revealed no significant diagnostic benefit compared to standard testing, is in agreement and further supports the results obtained in the first meta-analysis mentioned (1.1.). We found that CEA and cytology are more accurate than *KRAS* for diagnosis, confirming the limitations of molecular analysis in PCF, even using NGS in a cohort of surgical patients in which malignant and mucinous pre-malignant cysts predominate.

Our meta-analysis and original work allow us to conclude that, for the present time molecular analysis should not be recommended as a first line test in clinics. Its cost-efficacy must be further evaluated in real-life scenarios with PCF obtained by EUS-FNA prior to decision making.

Moving from genomics to metabolomics we explored the value of glucose level in PCF of mucinous and non-mucinous cysts and compared it to CEA level in a second study (2.2.).

Recently, some studies[84,85,86,127] have suggested that glucose is an alternative to CEA for mucinous cyst diagnosis. The rationale may rely on a phenomenon of reprograming the energy metabolism, with increased glucose uptake, denominated as the Warburg effect.[128] In the 1920s the biochemist Otto Warburg and colleagues observed that tumors took up enormous amounts of glucose, compared to surrounding tissue. Cancer cells rewire their metabolism as part of the recognized hallmarks of cancer[129], in order to promote growth, survival, proliferation, and maintenance, increasing glucose uptake and catabolism of glucose to lactate, which became known as the Warburg Effect.[128] Implications in health and disease continue to emerge, and the present days are considered a "renaissance" period for metabolomics research.[130]

Although the hallmarks of cancer have helped to gain insight into diagnostic and therapeutic targets, so far they have not translated into prevention. The future of prevention may be in the understanding of the hallmarks of normal cells and of ageing and relating them to the hallmarks of cancer, to break the linkage.[131] For this matter, pancreatic mucinous cysts, which are precursors of PDAC and increase with ageing, offer an excellent model.

We speculate that in mucinous cysts, low glucose levels may result from changes in cell metabolism, with a readjustment of cellular metabolism through glycolysis, regardless of oxygen availability, the so-called Warburg effect. These changes in cell metabolism may be determinant for transformation and tumor progression.[132] This breakthrough has been the basis for much research, including the widely-used cancer detection method - the positron emission tomography (PET) scan. The PET scan uses radioactive isotopes relying on the fact that cancer cells exhibit higher rates of glycolysis, to pinpoint tumors with advanced imaging tools, including malignancy in IPMNs and PCLs.[133]

To study the accuracy of PCF glucose using a glucometer and compare it to CEA level (2.2.) we evaluated 82 PCF samples obtained by EUS-FNA of different cyst types. The median glucose levels (interquartile range) were 19 mg/dL (19-19) in mucinous and 105 mg/dL (96-127) in non-mucinous cysts ($p<0.0001$). We specific a glucometer reading <20 mg/dL as 19 mg/dL. The median CEA level was 741 ng/mL (165-28567) in mucinous and 9 ng/mL (5-19) in non-mucinous cysts ($p<0.0001$). For mucinous cyst diagnosis, a CEA >192 ng/mL had a sensitivity of 72% (95%CI: 51-88), a specificity of 96% (95%CI: 82-100), and ROC analysis showed an AUC of 0.84 (95%CI: 0.73-0.96), while glucose<50 mg/dL had a sensitivity of 89% (95%CI: 72-98), a specificity of 86% (95%CI: 67-96), and an AUC of 0.86 (95%CI: 0.75-0.97). Pseudocysts presented low glucose, identically to mucinous cysts, with CEA allowing the differential diagnosis.

Our study showed that glucose level measured by a current glucometer was accurate for mucinous cyst diagnosis, as these cysts present a significantly lower glucose level than non-mucinous cysts. Additionally, almost a quarter of mucinous samples displayed a reading error in the glucometer, due to increased viscosity, pointing to the diagnosis. Pseudocysts were an exception, with low glucose levels, although being non-mucinous cysts.

Our results suggest that in clinical practice on site measurement of glucose at time of EUS-FNA, using a standard glucometer may replace CEA in mucinous cysts diagnosis, especially in small lesions with limited amount of PCF. Certainly, the insignificant cost and the technical simplicity of "in room" analysis, with no processing or storage required, is highly encouraging for its generalized use in clinical practice. To confirm our preliminary findings, large multicenter validation studies are necessary. Also, these future trials should explore clinical impact in patient outcomes, namely number of unnecessary surveillances that would be stopped, and, more importantly, number of surgeries of benign lesions avoided. The additional value of glucose in combination with cytology, in order to maximize the diagnoses of mucinous malignant and/or significant cysts, is also warranted.

In order to further elucidate these preliminary results we started a meta-analysis comparing glucose and CEA accuracies for diagnosis of mucinous cysts that was registered in PROSPERO (CRD42020163366). In addition, other methodologies that may allow the non-invasive diagnosis of PCLs in the future, such as PCF metabolomics using magnetic resonance spectroscopy (MRS), are already being explored by our group.

3. Original Research for Malignant Cyst Diagnosis

Our research on the diagnosis of malignant/high-risk cysts encompassed two retrospective cohort studies concerning repeating EUS-FNA in selected PCLs and its role in small cysts (<3 cm), as well as two exploratory studies to improve the diagnosis of cystic NETs and mucinous high-risk/malignant cysts.

First, we reviewed a cohort of patients with a second EUS-FNA and evaluated the added value of a second procedure in changing cyst diagnosis or management (3.1.).

In a cohort of 203 PCLs that were evaluated by EUS-FNA, surveillance was decided in 128 (63%). The data of 105 (82%) patients with a single EUS-FNA were compared with 23 (18%) with two EUS-FNAs during surveillance. Patients were younger in this latter group (p=0.055), whereas CEA levels were marginally higher (p=0.078) and mass or nodules were more frequent (p=0.006). Four patients were referred for surgery (p=NS) after two EUS-FNAs [2 NETs, 1 IPMN-HGD and 1 MCN-LG]. A high correlation of CEA level between two consecutive EUS-FNAs (r2=0.945, p<0.01) was observed, with a reclassification (cut-off level of 192 ng/mL) in 2 patients only. Of 4 patients with a second EUS-FNA with conclusive cytology, 2 had NETs confirmed in the surgical specimen.

Our results support repeating EUS-FNA in surveillance of selected PCLs, as it resulted in a change in management toward surgery in approximately 20% of the patients. CEA had a high correlation between EUS-FNAs, rarely changing cyst classification. Our data drew attention to the limited accuracy of morphology for malignant risk stratification of PCLs established in the AGA guidelines[81], particularly for cystic NETs. Moreover, the same AGA recommendation stating that asymptomatic cysts with a very low risk of malignant transformation (presumed SCAs) do not require further evaluation is questioned by our results, as 2 NETs were misdiagnosed in the first EUS-FNA. The revision of Fukuoka guidelines[79] and the new guidelines of the ACG[97] are more cautious, with EUS-FNA recommended in the scenario of WFs or unclear cyst diagnosis, respectively, possibly improving EUS-FNA diagnostic performance.

Although pancreatic surgery still carries high morbidity and mortality and should be reserved for high-risk lesions, PCLs also represent a rare opportunity for early pancreatic cancer diagnosis and possible cure. Despite being rare, cystic NETs should not be overlooked, and the long-term impact of repeating an EUS-FNA particularly in lesions with one or even no risk stigmata and CEA <192 ng/mL, deserves further investigation.

The results of this first study on the diagnosis of malignant/high-risk cysts opened two new lines of research. First, to assess the limited sensitivity of imaging morphology for PCLs malignant risk stratification using current guidelines, namely the role of EUS-FNA for PCF analysis in small PCLs (<3 cm). Second to conduct an exploratory study looking for putative biomarkers for cystic NETs diagnosis.

Current guidelines recommend evaluation of PCLs with EUS-FNA if WFs (size ≥3 cm, mural nodule, or Wirsung dilation) are present. We hypothesized that the superior imaging quality of EUS with additional FNA for PCF analysis, including CEA and cytology, could improve classification and clinical decision even in small cysts (3.2).

For this purpose, we reviewed the results of EUS-FNAs performed between 2007 and 2016 in 115 patients with PCLs <3 cm and evaluated PCF analysis for detection of malignancy and surgical triage. In 19 patients that underwent surgery, 7 had malignant lesions, 8 pre-malignant, and the remaining 4 had benign lesions. Mass/mural nodules were present in 27% of the cysts, CEA level was higher than 192ng/mL in 39.4% of the patients, with 35% of informative cytological samples. Nevertheless, biochemical, and cytological PCF analysis improved the diagnostic performance of EUS imaging alone, from an AUC of 0. 6 to an AUC of 0.8.

Our results confirmed that even in lesions without worrisome features, 2 out of 5 resected lesions were high-risk/malignant lesions, which is quite similar to series including lesions larger than 3 cm.[72,134,30,135] Additionally, EUS-FNA allowed the diagnosis of benign cysts, allowing to stop surveillance in 1 out of 5 patients.

In summary, when discussing the role of EUS-FNA in pancreatic cysts smaller than 3 cm, one can support either side of the coin. On the one hand, we found most PCF analysis inconclusive (more than 40% of CEA levels between 5 and 192ng/mL, and overall, two thirds of acellular samples) making EUS-FNA an invasive and often unhelpful technique. On the other hand, EUS-FNA allowed the diagnosis of high-risk lesions in some patients who would otherwise be surveilled, potentially improving outcome and cost-effectiveness of the program. Mass/nodules were helpful for malignancy diagnosis, despite being absent in cystic NETs and cystic PDACs and often present in non-high-risk IPMNs.

As the performance of any isolated marker is imperfect, according to our results, combining clinical, morphological, biochemical, and cytological data significantly improves diagnosis of malignancy even in lesions smaller than 3 cm.

In order to look for putative biomarkers for the diagnosis of cystic NETs, we started by reviewing previous publications. There were two studies evaluating the accuracy of Chromogranin A (CroA) in PCF with discrepant results.[17,83]

Supported by this literature review we started an exploratory study evaluating CroA and NSE in PCF of 16 PCLs (3.3.).

PCF samples were selected from the EUS-FNA registry of our Hospital. To standardize PCF analysis, we measured CroA (CGA-RIACT®, Cisbio Bioassays, France), and NSE (LIAISON®XL NSE, DiaSorin, Italy) in 0.25 mL of frozen PCF. The 16 PCF samples included 4 cystic NETs, 7 benign (4 serous cystadenomas, 3 pseudocysts), 3 pre-malignant (3 IPMNs), and 2 malignant (1 acinar cell tumor, 1 IPMN-HGD) cysts. Amylase, CEA, and glucose levels were not significantly different in cystic NETs. In contrast, CroA and NSE levels were higher in cystic NETs (median values of 319.2 ng/mL and 412.7 ng/mL, respectively) than in other cysts (median values <42 ng/mL and <0.8 ng/mL, respectively), p=0.005 and p=0.002, respectively. In the diagnosis of cystic NETs, the AUC was 0.94 (95%CI: 0.81-1)

for CroA and 1 (95%CI: 1-1) for NSE, with optimal cut-off values >149 ng/mL for CroA and >99 ng/mL for NSE.

Our preliminary results suggest that elevated levels of CroA and NSE in PCF accurately distinguish cystic NETs from other PCLs. According to our study, determination of CroA and NSE in PCF may complement a non-diagnostic cytology result, especially in cysts that typically present a low CEA and/or high glucose levels, which may erroneously suggest a benign cyst.

In agreement with our results, a previous publication by Levy *et al.*[83] evaluating CroA A and NSE in 28 PCF samples, including 5 cystic NETs, found higher median values in NETs than in other cystic lesions. On the other hand, Oruç N *et al.*[17] evaluated Cro A in 53 PCF samples and found no difference in Chromogranin A between different cyst types but their series included 2 cystic NETs only.

In our study no clinical or imaging features could differentiate cystic NETs, except a tendency for younger age, in NET patients *versus* 61 in non-NETs (p=0.072) and a predominant location in the pancreatic body in 3/4 cysts (p=0.08). In our series, only 2/4 (50%) of cysts showed these features. This imaging dilemma makes cytology a cornerstone in cystic NETs diagnosis and targeting the cyst wall during FNA is recommended. In our study, 50% (2/4) of cystic NET FNAs were acellular, compared to 21% (4/19) in a study by Yoon *et al.*[136], and 21% (5/24) in a study by Ho *et al.*[137]. This may be explained by our reduced number of cystic NETs. Standard PCF biochemical analysis in cystic NETs shows low CEA (<192 ng/mL) and high glucose (>50 mg/dL), which are non-diagnostic and identical to other more common non-mucinous cysts, such as SCAs and pseudocysts. This common biochemical profile and a non-diagnostic cytology may erroneously suggest a benign cyst, whereas in fact we are dealing with a potentially malignant cyst, which emphasizes the need for accurate biomarkers for these cysts.

More extensive studies performed in other centers, if possible using the same assays for diagnosis, may validate our findings and lead to a uniform interpretation of these promising test results.

In a second exploratory study we evaluated methylation changes in the *GNAS locus* in PCF for diagnosing malignant cysts and monitoring cancer progression (3.4.).

DNA methylation commonly refers to the covalent addition of a methyl (−CH3) group from the s-adenosylmethionine to the fifth carbon of the cytosine base (5mC), which is catalyzed by DNA methyltransferases. It is extensively demonstrated that DNA methylation plays a key role in chromosomal stability, gene expression, genome imprinting, and transcriptional silencing of foreign DNA fragments.[138]

Pathological alterations in DNA methylation patterns are described in a variety of diseases, including cancer. Unlike genetic changes, DNA methylation is heavily influenced by subtle modifications in the cellular microenvironment. Aberrant DNA methylation in cancer is

involved in the alteration of a large number of oncological pathways with relevant theragnostic utility.

IPMNs present *GNAS* mutations with PDACs harboring *GNAS* mutations originating from IPMNs. *GNAS* is a complex imprinted *locus* that produces 5 transcripts regulated by differential methylated regions (DMRs), *NESP55*, *GNASAS*, *GNASXL*, *GNAS1A,* and *GNAS*. In this study we evaluated if methylation changes in the DMRs of *GNAS locus* contributed to malignant progression of pancreatic cysts. *GNAS locus* methylation was analyzed in 52 archival samples of PCF, obtained by EUS-FNA, using *methylation specific–multiplex ligation dependent probe amplification* (MLPA).

Methylation changes at *NESP55, GNASAS, GNAS1A*, and especially *GNASXL* were more frequent in malignant cysts, thereby being a useful marker for the diagnosis of malignancy. A combined variable defined as "*GNAS locus* methylation changes" was significantly associated with malignancy (6/8 malignant cysts and only 2/20 benign cysts) and improved classification.

This was the first study to identify methylation changes in the *GNAS locus,* improving the diagnosis of malignant PCLs and suggesting a role for progression to PDAC.

Hypermethylation with *GNAS locus* gain of function and its possible association with malignant progression remains poorly understood, but *GNAS* oncogenic potential appears unquestionable. Hypermethylation in both maternally (*NESP55*) and paternally (*GNASXL*) derived promoters, and therefore overall increase of methylation in these two DMRs, was detected exclusively in PDACs, further suggesting a role of *GNAS* in malignant progression. Interestingly, the detection of exonic *GNAS* hypomethylation in the pancreatic NET is in agreement with the recent findings showing that pancreatic NETs are genetically and phenotypically related to pancreatic ductal adenocarcinoma, having a closer relationship to ductal adenocarcinomas than to neuroendocrine tumors G3.[139]

An analysis of loss of heterozigoty (LOH) in the *GNAS locus* would be needed to evaluate uniparental disomy (UPD) associated copy-neutral LOH (which can often be segmental) and investigate whether some of these methylation alterations may indeed reflect epigenetic alterations, or could instead be explained by acquired UPD, at least in part. Nevertheless, independently of their cause (epigenetic or acquired UPD), the resulting methylation alterations detected in the *GNAS locus* DMRs appear to be related to malignant progression and may improve malignant cyst diagnosis.

Our pilot study requires further confirmation in a validation cohort, but propels the current epigenetic landscape of pancreatic cysts, possibly offering an opportunity for early diagnosis for pancreatic cancer.[140,141]

4. Performance of the Biomarkers Evaluated in this Dissertation

In Table 6 we compare the diagnostic performance of the different biomarkers studied.

PCF glucose level, evaluated with an *on-site* glucometer, is easy, immediate, and requires minimal PCF. It had the highest sensitivity and accuracy for mucinous cyst diagnosis, followed by CEA level, and thirdly by *KRAS* mutational analysis.

GNAS locus methylation changes performed better than cytology for malignancy diagnosis. Although evaluated in a small cohort of PCLs in an exploratory study, CroA and NSE seem to be promising biomarkers for diagnosis of cystic pancreatic NETs.

Finally, as in recent publications[29], it appears from the analysis of our data that composite markers (combining multiple individual parameters into a single marker) may offer additional advantages for diagnosis of PCLs.

Table 6. Biomarkers evaluated in this dissertation and their performance for diagnosis of pancreatic cystic lesions, including mucinous, malignant, and cystic NETs.

Test	Positive result or cut-off	Sensitivity,% (95% CI)	Specificity,% (95% CI)	AUC
Mucinous *vs* non-mucinous				
EUS – Imaging	Mucinous: all cases, except cysts suggesting SCAs or with no septs or nodules and features of pancreatitis.	53 (28-77)	83 (52-98)	0.68 (0.48-0.88)
CEA	>192 ng/mL	72 (51-88)	96 (82-100)	0.84 (0.73-0.96)
KRAs and *GNAS* mutations	*KRAS* (exons 2 and 3) *GNAS* (exons 8 and 9)	50 (25-75)	100 (72-100)	0.72 (0.52-0.92)
Glucose	<50 mg/dL	89 (72-98)	86 (67-96)	0.86 (0.75-0.97)
Malignant *vs* non-malignant				
Cytology	Malignant or atypical cells defined high-risk malignant cysts.	67 (30-93)	90 (68-99)	0.79 (0.57-1)
GNAS Methylation Changes	DMR hypermethylation or *GNAS* intragenic hypomethylation	75 (35-97)	93 (78-99)	0.97 (0.9-1)
NET *vs* non-NET				
CroA	>149 ng/mL	100 (40-100)	92 (62-100)	0.94 (0.81-1)
NSE	>99 ng/mL	100 (100-100)	100 (100-100)	1 (1-1)

CI, confidence interval; AUC, area under the receiver-operating characteristics curve; CEA, carcinoembryonic antigen; CroA, chromogranin A; NSE, neuron specific enolase; *GNAS*, Guanine nucleotide binding protein, alpha stimulating; NET, neuroendocrine tumor

5. A Revised Organogram for the Diagnosis of Pancreatic Cystic Lesions

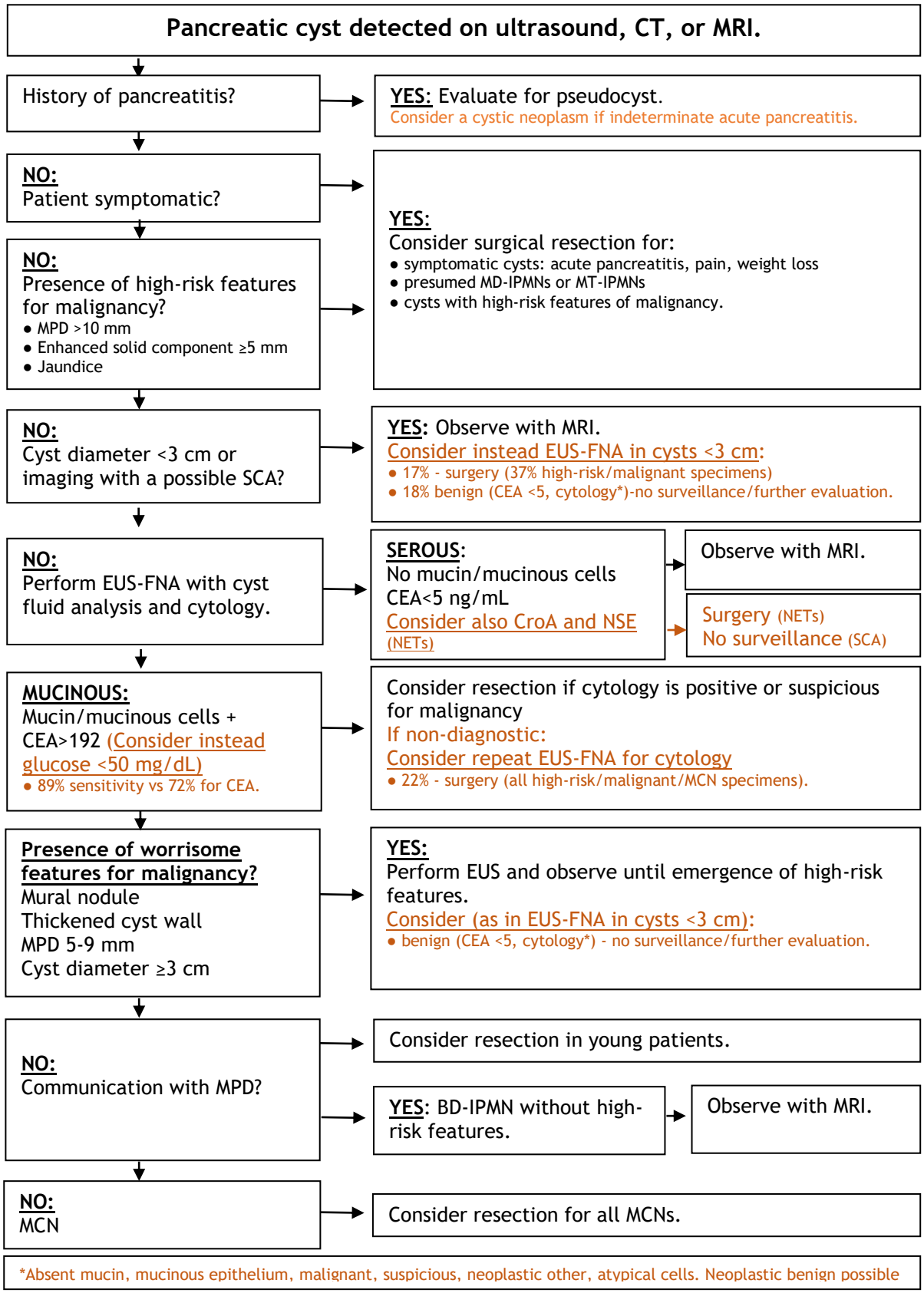

Figure 8. Organogram for evaluation and management of pancreatic cystic lesions, including the variations established in this dissertation. The proposed changes are displayed in orange.
Addapted from World Gastroenterology Organization global Guideline, 2019[142].

In Figure 10 we propose a revised organogram for diagnosis of PCLs in patients fit for surgery and willing to undergo a pancreatic resection.

Considering our original data and our analysis of biomarkers for classification and risk assessment of pancreatic cystic neoplasms, we are in position to suggest a few changes to current evaluation, which are displayed in orange. Initially, the evaluation of glucose level instead of CEA for mucinous cyst diagnosis and consider measuring CroA and NSE levels for diagnostic evaluation of cystic NETs. Additionally, repeating EUS-FNA in selected PCLs and performing EUS-FNA in PCLs smaller than 3 cm may further restrict surgery to high-risk/malignant cysts and eventually, allow to stop surveillance in a significant portion of patients with benign cysts.

6. Future studies

We confirmed that somatic mutations in *KRAS* are early events in IPMNs and MCNs that can be detected in PCF, but the techniques available for molecular analysis are complex, expensive, burdensome, and time-consuming. Furthermore, to obtain PCF for analysis, EUS-FNA is required, an invasive procedure that includes rare, but possible adverse events.

With these drawbacks in mind, two new opportunities to approach PCLs were identified and are underway. One is to evaluate a digital microfluidic (DMF) platform for easy and immediate *KRAS* mutational analysis in PCF, and the other is to evaluate PCF metabolomics by MRS in order to study its discriminating capacity of different PCLs, potentially allowing a non-invasive diagnostic approach of PCLs in the future.

Chapter VI.

Conclusions and Final Remarks

The original and review work presented in this dissertation allow us to propose some adjustments to the flowchart of PCLs analysis published so far, according to current guidelines, which may become relevant for clinical decisions.

In order to recognize mucinous cysts that require surveillance due to malignancy risk, we provide evidence that the diagnosis of PCLs can be optimized and simplified by using a glucometer with immediate "in-room" glucose level determination. In contrast, we found that molecular analysis for *KRAS* mutation in PCF did not improve the diagnostic accuracy of standard CEA. To identify high-risk cysts and as compared to genetic analysis, micro forceps analysis has a greater diagnostic yield and may be useful as a second line test.

Repeating EUS-FNA for additional cytology and measurement of CroA and NSE in PCF improves NETs diagnosis in cases of a false-negative cytology result related to scant cellularity. Also, EUS-FNA in small pancreatic cysts (<3 cm) may be justified as it results in the identification of both malignant lesions with surgical indication and benign cysts that should be safely discharged from surveillance.

Finally, an exploratory study of methylation changes at *GNAS complex locus* in PCF reveals promising results for the diagnosis of high-risk and malignant cysts. This revolutionary new data deserves additional research in order to validate this promising finding.

Our proposal of a simplified EUS-FNA diagnostic strategy is supported on a patient population representative of current clinical practice, in which most lesions are benign or of low risk, generally requiring surveillance, and only rarely calling for surgical resection. Our main goal should be to restrict surveillance to pre-malignant cysts and to confirm malignancy before surgery in mucinous cysts and cystic NETs.

PCF analysis is a field of research with tremendous clinical potential in which the identification of new biomarkers may help to individualize decisions on whether to operate, surveil, or discharge patients with PCLs.

Although obvious limitations still exist, our work sheds light on how to manage different categories of patients. In this individualized balance, fitness for surgery, life expectancy, and location of the cyst that defines the type of pancreatic resection, may become as relevant for clinical decisions as the cyst itself. In fact, current guidelines are fundamentally "cyst-centered".

Future research in this field, aiming at validating a flowchart which combines patient and cyst-related factors, needs to consider several dimensions such as disease progression, burden of overtreatment, costs involved, and potential adverse events.

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Pancreatic neuroendocrine carcinomas reveal a closer relationship to ductal adenocarcinomas than to neuroendocrine tumors G3. Hum Pathol. 2018;77:70-79. doi: 10.1016/j.humpath.2018.03.018. Epub 2018 Mar 26.

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141. Majumder S, Taylor WR, Yab TC, Berger CK, Dukek BA, Cao X, et al. Novel Methylated DNA Markers Discriminate Advanced Neoplasia in Pancreatic Cysts: Marker Discovery, Tissue Validation, and Cyst Fluid Testing. Am J Gastroenterol. 2019;114(9):1539-1549. doi: 10.14309/ajg.0000000000000284.

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Addendum

1. Europass CV

europass Curriculum vitae

PERSONAL INFORMATION

Sandra de Jesus Reis Faias Antunes

Largo Costa Pinto, 2805-265 Almada (Portugal)
(+351)914310209
sandrarfaias@hotmail.com

Sex Female | Date of birth 17/06/1973 | Nationality Portuguese

POSITION Gastroenterologist

WORK EXPERIENCE

01/03/2006–Present
Gastroenterologist
Instituto Português de Oncologia de Lisboa de Francisco Gentil, Lisboa (Portugal)

EUS - Radial and Linear (300 procedures/year)
EGD (400 procedures/year)
PEG (200 procedures/year)
Colonoscopy (500 procedures/year)
GI consults (1000/year)
Inpatients (variable)

01/03/2006–Present
Gastroenterologist
Hospital da Luz - Torres de Lisboa, Lisboa (Portugal)

- EGD (200/year)
- Colonoscopy (300/year)
- GI consultas (600/year)

01/01/2016–15/08/2016
Gastroenterologist and GI Unit Coordinator
Hospital Lusíadas, Lisboa (Portugal)

-EGD, Colonoscopy, PEGs, EUS, GI Consults, GI inpatients.

EDUCATION AND TRAINING

01/09/1992–25/06/1998
Medical Degree
Faculdade de Medicina- Universidade de Lisboa, Lisboa (Portugal)

Final: 16,33

01/01/1999–30/12/2000
General Medical Training
Hospital Garcia de Orta, Almada (Portugal)

08/10/2000–08/10/2000
National Exam for Medical Specialities
Medical Training Comission, Lisbon (Portugal)

01/01/2001–30/12/2005
Gastroenterology Specialist
Instituto Português de Oncologia de Lisboa de Francisco Gentil, Lisboa (Portugal)

Director: C Nobre leitão

02/01/2003–03/01/2003 **USMLE 0- 631-542-8**

Educational Commission for Foreign Medical Graduates

USMLE CERTIFICATE (2003)

Basic Medical Science Examination

Clinical Science Examination

Clinical Skills Assessment

TOEFL test

01/07/2004–01/06/2005 **EUS Advanced Training "Hands-on"**

Digetive Disease Center, Medical University of South Carolina, Charleston (United States)

Director: Peter B Cotton

EUS (Brenda Hoffman, Robert Hawes, Joseph Romagnulo)

Clinical Research (EUS), Clinical Nutrition (Mark DeLegge), Endoscopic Capsule (Brenda Hoffman), GI Motility (Donald O Castel)

07/07/2008–25/07/2008 **Observational Training EUS and Therapeutic Endoscopy**

Institut Paoli-Calmettes, Marselha (France)

Director: Marc Giovanini

25/02/2014–28/02/2014 **Master Class Program on Endoscopic Ultrasound (Step 1)**

Hospital Universitario Santiago Compostela, Santiago Compostela (Spain)

Coordinator: Julio Iglesias-Garcia

20/05/2014–22/05/2014 **Master Class Program on Endoscopic Ultrasound (Step 2)**

Hospital Universitario de Santiago de Compostela, Santiago Compostela (Spain)

07/04/2014–04/05/2014 **ESGE Fellowship Grant 1 (ERCP)**

Digestive Endoscopy Unit, Polliclinico A. Gemelli, Roma (Italy)

Director: Guido Costamagna

01/10/2013–31/01/2016 **Advanced Training in ERCP**

Hospital de Santa Maria, Lisboa (Portugal)

Dr. Rui Palma

"Hands on" training

29/04/2016–25/11/2016 **Healthcare Management Program 4th Edition**

Católica Lisbon of Business and Economics, Lisboa (Portugal)

01/09/2016–Present **PhD Student**

Faculdade de Ciência Médicas, Universidade da Beira Interior, Covilhã (Portugal)

"Biomarkers for diagnosis and risk assessment of pancreatic cystic neoplasms".

europass Curriculum vitae

PERSONAL SKILLS

Mother tongue(s): Portuguese

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C1	C2	C1	C1	C1
	First Certificate in English - Grade B 199 TOEFL -2003				
French	A2	A2	A1	A1	A2
Spanish	B1	B1	A2	A2	A2

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages

Communication skills: Good communication skills gained through several training periods in several institutions in Portugal and abroad

Organisational / managerial skills: Leadership (currently responsible for the PEG Ambulatory Services, Instituto Português de Oncologia de Lisboa de Francisco Gentil)

Job-related skills: Mentoring skills (Currently the responsible for GI training in PEGs and EUS in Instituto Português de Oncologia de Lisboa de Francisco Gentil)

Digital skills

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem-solving
Proficient user	Independent user	Basic user	Basic user	Basic user

Digital skills - Self-assessment grid

- good command of office and spss

ADDITIONAL INFORMATION

Honours and awards

1. BEST CLINICAL CASE (Congresso Nacional de Gastrenterologia, Porto 2003)

Faias S., Lage P., Sache F., Fidalgo P., Pinto A., Fonseca I., Nobre Leitão C. Pemphigus Vulgaris com envolvimento esofágico isolado: A raridade de um caso clínico. **GE – Jornal Português de Gastrenterologia (suplemento) 10: 74, 2003**

2. BESTPOSTER (Congresso Nacional de Gastrenterologia, Porto 2003)

Faias S., Midões Correia J., Chaves P., Claro I., Dias Pereira A., Meneses Costa J., Nobre Leitão C. Importância no prognóstico dos valores pré-operatórios de CEA e CA 19-9 no Cancro Gástrico. **GE – Jornal Português de Gastrenterologia (suplemento) 10: 94, 2003**

3. BEST ABSTRACTS (11th United European Gastroenterology Week, Madrid 2003)

Faias S., Midões Correia J., Ferreira S., Dias Pereira A., Chaves P., Nobre Leitão C. Recurrence of Adenomas in the Colon and Rectum. Prospective Analysis of 566 Cases. **Gut (supplement N II) 35: A 190, 2003**

4. 2º PRIZE - BEST COMUNICAÇÃO CLÍNICA (Congresso Nacional de Gastrenterologia, Vilamoura 2007)

Rosa I, Vinga S, Oliveira C, Pereira da Silva J, Mão-de Ferro S, Faias S, Midões J, Pinto A, Chaves P, Viveiros CF, Fidalgo P, Oliveira AG, Soares J, Nobre Leitão C. Pode a polipectomia extinguir a

carcinogénese colo-rectal? **GE – Jornal Português de Gastrenterologia (suplemento) 14: 15, 2007**

5. 1º PRIZE GUPUGE: Best EUS presentation in Portuguese Digestive Week 2012, Porto-Portugal

Fidalgo C, **Faias S,** Pereira Silva J, Fonseca R, Dias Pereira A. Quistos do pâncreas avaliados por Ecoendoscopia: Punções, operações e complicações. **GE – Jornal Português de Gastrenterologia (suplemento) 19: 77, 2012.**

6. CLUBE PORTUGUÊS DO PÂNCREAS: BEST Apresentação de Patologia Pancreática – Semana Digestiva 2014, Estoril-Portugal

Faias S, Pereira Silva J, Fonseca R, Andre S, Dias Pereira A. Tumores quísticos do pâncreas operados: Acuidade dos critérios revistos de Sendai na Avaliação de Malignidade. **Livro de resumos do Congresso** http://semanadigestiva.pt/wp-content/uploads/2013/04/Programa-Definitivo-SD2014.pdf)

7. PRIZE GE - Jornal Português de Gastrenterologia – Melhores resumos apresentados por internos da especialidade na Semana Digestiva 2014, Estoril 2014

Rodriges RV, Faias S, Moleiro J, Serrano M, Femenia M, Severiano S, Machado V, Dias-Pereira A. Os doentes com tumores da cabeça e pescoço submetidos a quimioradioterpia definitiva têm disfagia prolongada e necessitma de PEG profilática. **Livro de Resumos da Semana Digestiva 2014 P29**. (http://semanadigestiva.pt/wp-content/uploads/2013/04/Programa-Definitivo-SD2014.pdf)

8. Premio CPP 2019. Melhor trabalho sobre patologia pancreática.

Faias S, Duarte M, Chaves P, Cravo M, Dias Pereira A, Albuquerque C.

Methylation changes at the GNAS imprinted locus in pancreatic cystic neoplasms: role in the diagnosis of mucinous and malignant cysts? Resumo da Semana Digestiva 2019

10.Poster Champ of United European Gastroenterology Week 2019

Faias, S; Pereira da Silva, J; Marques, I; Fonseca, R; Cravo M; Chaves, P; Dias Pereira, A.

Relevance of Endoscopic Ultrasound with Fine Needle Aspiration in Pancreatic Cystic Lesions Smaller than 3 cm. A retrospective study.

Publications

1. Marcelino P., **Faias S.**, Fernandes A. P., Marum S., Palmeiro Ribeiro J.

Deplecção Transitória e Rara de Linfócitos CD_4: A Propósito de um Caso Clínico.

Anamnesis 2002; 109: 22-3.

2. S. Faias, A. Alberto Santos, C. Nobre Leitão.

Obstrução Duodenal e Biliar por Neoplasia da Cabeça do Pâncreas: Paliação Endoscópica (Pancreatic Head Carcinoma with Duodenal and Biliary Obstruction: Endoscopic Palliation).

GE – Jornal Português de Gastrenterologia 2004; 11: 84-88.

3. Faias S., Midões Correia J., Piteira Barros F., Cunha J.F., Mendes de Almeida J.M., Costa Rosa J.,Nobre Leitão C.

Tumor Sólido Pseudopapilar do Pâncreas: Apresentação de um caso clínico e revisão da literatura. (Solid Pseudopapillary Tumor of the Pancreas: Case report and review of the literature).

GE – Jornal Português de Gastrenterologia 2004; 11: 213-216.

4. Faias S., Venâncio J., Barata A., Bettencourt A., Limbert M., Cunha J.F., Mendes de Almeida J.C., Fidalgo P., Meneses Sousa J., Lacerda A., Nobre Leitão C.

Valor da Informação do Toque Rectal, Tomografia Computorizada e Ecografia Endo-Rectal na Decisão Terapêutica do Cancro do Recto: Estudo Randomizado.(Rectal Cancer Treatment Decision: A Randomized Evaluation of Multimodalaty Staging).

GE – Jornal Português de Gastrenterologia 2004; 11: 191-8.

5. Sandra Faias, MD , Pedro Lage, MD , Fernanda Sachse, MD , Anabela Pinto, MD , Paulo Fidalgo, MD , Isabel Fonseca, MD, PhD , Carlos Nobre-Leitão, MD, PhD.

Pemphigus Vulgaris with Exclusive Involvement of the Esophagus: Case Report and Review.

Gastrointestinal Endoscopy 2004; 60: 312-5.

6. Sandra Faias, Jaime Midões Correia, Paula Chaves, C. Nobre Leitão.

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Pólipos do Cólon e Recto. Diagnóstico e Vigilância.

Cadernos de Formação em Gastrenterologia.

GE – Jornal Português de Gastrenterologia 2004; 11: 144-150.

7. S. Faias, R. Sousa, A. Alberto Santos, P. Chaves, C. Nobre Leitão.

Metástase Pancreática de Tumor da Mama: Causa Rara de Icterícia Obstrutiva. (Pancreatic Metastases from Breast Cancer: An unusual cause of biliary obstruction).

GE – Jornal Português de Gastrenterologia 2004; 11: 318-21.

8. Sandra Faias, Marília Cravo, Isabel Claro, A. Dias Pereira, Nobre Leitão C.

Gastrostomia Endoscópica Percutânea pelo Método do Introdutor (Percutaneous Endoscopic Gastrostomy with the Introducer Method)

GE – Jornal Português de Gastrenterologia 2005; 12: 22-6.

9. Sandra Faias, Marília Cravo, Isabel Claro, Pedro Lage, C. Nobre-Leitão

High Rate of Percutaneous Endoscopic Gastrostomy (PEG) Site Infections due to Oropharyngeal Colonization

Dig Dis and Sci 2006; 51(12) 2384-8

10. Sandra Faias, Gregory Buck, Mark DeLegge

Peritonitis after Percutaneous Endoscopic Gastrostomy and Jejunostomy: Where There is Smoke, There May Not be Fire

Endoscopy 2006; 38 (7): 745-8

11. Correia M, Cravo M, Marques-Vidal P, Grimble R, Dias-Pereira A, **Faias S**, Nobre-Leitão C

Serum Concentrations of TNF-alpha as a surrogate marker for malnutrition and worse quality of life in patients with gastric cancer

Clin Nutr 2007; 26(6): 278-35

12. Moleiro J, **Faias S**, Fidalgo C, Serrano M, Pereira AD.

Usefulness of Prophylactic Percutaneous Gastrostomy Placement in Patients with Head and Neck Cancer Treated with Chemoradiotherapy.
Dysphagia. 2016 Feb;31(1):84-9.

13. Moleiro J, **Faias S,** Afonso A.

Esophageal Stricture of an Unusual Etiology.
Clin Gastroenterol Hepatol. 2016 Jun;14(6):e59-e60.

14. Rodrigues RV, **Faias S,** Fonseca R.

Giant Pancreatic Cyst: An Unusual Entity.
GE Port J Gastroenterol. 2016 Apr 3;23(6):314-315.

15. Mota PJL, Dionísio J, Duro da Costa J, **Faias S.**

Flexible Bronchoscope Use by Pulmonologists in Collaboration With Gastroenterologists for Placement of Percutaneous Endoscopic Gastrostomy Via Transnasal Route in Patients With Head and Neck Cancer: A 10-Year Experience in a Single Cancer Institution.
J Bronchology Interv Pulmonol. 2018 Jan;25(1):73-77.

16. Faias S, Duarte M, Albuquerque C, da Silva JP, Fonseca R, Roque R, Dias Pereira A, Chaves P, Cravo M.

Clinical Impact of KRAS and GNAS Analysis Added to CEA and Cytology in Pancreatic Cystic Fluid Obtained by EUS-FNA.
Dig Dis Sci. 2018 Sep;63(9):2351-2361.

17. Faias S, Pereira L, Luís Â, Cravo M, Dias Pereira A, Torres J.

KRAS in Cyst Fluid Obtained by Endoscopic Ultrasound-Fine-Needle Aspiration in Pancreatic Cystic Lesions: A Systematic Review and Meta-analysis.
Pancreas. 2019 Jul;48(6):749-758.

18. Faias S, Pereira L, Luís Â, Chaves P, Cravo M.

Genetic testing vs microforceps biopsy in pancreatic cysts: Systematic review and meta-analysis.

World J Gastroenterol 2019; 25(26): 3450-3467.

19. Lemos Garcia J, Mão-de-Ferro S, **Faias S,** Marques I, Pereira AD.

Spontaneous colonic lipoma expulsion after diagnostic colonoscopy: a peculiar occurrence.
Gastrointest Endosc. 2020;91(2):449-450. doi:10.1016/j.gie.2019.09.004

20. Faias S, Pereira L, Roque R, Chaves P, Torres J, Cravo M, Pereira AD.
Excellent Accuracy of Glucose Level in Cystic Fluid for Diagnosis of Pancreatic Mucinous Cysts.
Dig Dis Sci. 2019 Nov 9. doi: 10.1007/s10620-019-05936-5.

21. Currais P, **Faias S,** Francisco F, Sousa L, Gramacho J, Pereira AD.
Gastrocutaneous fistulas after PEG removal in adult cancer patients: frequency and treatment options
Surg Endosc. 2020;10.1007/s00464-020-07627-z. doi:10.1007/s00464-020-07627-z

Memberships
Portuguese Society of Gastroenterology
Portuguese Society of Digestive Endoscopy
European Society of Gastrointestinal Endoscopy
American Society of Gastrointestinal Endoscopy

Scholars
2003:
Sociedade Portuguesa de Endoscopia Digestiva
Núcleo Regional do Sul da Liga Portuguesa Contra o Cancro
Fundação Luso-Americana para o Desenvolvimento
2013:
ESGE (Fellowship Grant - Module1)
2015:
Bolsa do Clube Português do Pâncreas - Abbott
2018:
Bolsa da Sociedade Portuguesa de Endoscopia Digestiva

2. Patient Informed Consent

REGISTO DE QUISTOS DO PÂNCREAS DO IPOLFG

CONSENTIMENTO INFORMADO

Folha de Informação do Doente

Objetivo do Estudo:

1. **Criar um registo prospetivo de doentes com quistos o pâncreas submetidos a Ecoendoscopia com punção (EUS-FNA) no IPOLFG.**
2. **Fazer a vigilância clínica e imagiológica das lesões quísticas identificadas.**
3. **Colher e armazenar amostras de sangue e líquido quístico para identificar ou validar marcadores de malignidade nas lesões quísticas precursoras do adenocarcinoma do pâncreas (as neoplasias mucinosas papilares intra-ductais (IPMNs) e as neoplasias mucinosas quísticas (MCNs)).**

A sua participação no estudo é inteiramente voluntária. Se decidir não participar isso não influenciará os seus cuidados médicos ou a sua relação com o(s) seu(s) Médico(s) Assistente(s).

Resumo: Os quistos do pâncreas são achados incidentais frequentes pelo aumento da idade e pela utilização generalizada de métodos de imagem abdominal. Cerca de 10% dos indivíduos com mais de 70 anos têm quistos do pâncreas. A maioria são lesões precursoras do adenocarcinoma do pâncreas, especificamente os IPMNs e as MCNs.

É desconhecida taxa e as características dos quistos que progridem para lesões agressivas (displasia de alto grau e carcinoma invasivo). A progressão resulta das células da parede destes quistos sofrerem por uma série de alterações, podendo tornar-se malignas. A estas alterações celulares que são visíveis ao microscópio (exame citológico), chama-se displasia. Quanto mais alterada é a aparência das células, maior é o grau de displasia, que se classifica em 3 classes: baixo grau, grau intermédio e alto grau. Ao microscópio, a displasia de alto grau é igual a cancro, mas as células ainda não invadem o sistema ductal e por isso não conseguem espalhar-se pelo organismo e não são ainda consideradas verdadeiro cancro.

Estas lesões representam um dilema, pois o adenocarcinoma do pâncreas, mesmo em estádios precoces, tem um prognóstico reservado, sendo a sobrevida global aos 5 anos de 8%. Por outro lado, o tratamento cirúrgico das lesões precursoras tem morbilidades (50%) e mortalidades (5%) significativas mesmo em centros de elevado volume.

O nosso objetivo é detetar os quistos com displasia de alto grau. Se isto já fosse possível na atualidade, a solução seria operar os quistos imediatamente antes de passarem a carcinoma invasivo. Como ainda não temos essa capacidade, procuramos pelas características imagiológicas (TC e RMN) do quisto e da citologia do líquido quístico (EUS-FNA) com uma acuidade limitada tomar a decisão terapêutica.

Com este registo prospetivo pretendemos no futuro identificar as lesões precursoras de risco (DAG ou carcinoma invasivo), de forma operar apenas as lesões agressivas.

Todos os doentes com quistos do pâncreas avaliados por Ecoendoscopia ficarão registados numa base de dados, que inclui atualmente cerca de 250 doentes. Este registo de quistos do pâncreas será atualizado com informação clínica e imagiológica prospetivamente, de forma a definir a história natural dos quistos.

No líquido quístico e em sangue periférico pretende-se identificar biomarcadoresque permitam prever quais os quistos que irão desenvolver adenocarcinoma do pâncreas e tratá-los previamente.

Como vai funcionar o processo de colheita de material?

A participação no estudo não altera o procedimento diagnóstico habitual dos quistos do pâncreas por Ecoendoscopia, não implicando quaisquer procedimentos ou riscos adicionais, e não terá qualquer encargo económico adicional.

1. **Líquido quístico:**

Na sequência da Ecoendoscopia com punção necessária à avaliação diagnóstica do quisto pancreático de que é portador, será feita uma punção do quisto para obtenção de líquido. Este será analisado, conforme procedimento habitual para CEA e citologia.

O líquido remanescente (sobrante) após a análise habitual será armazenado e posteriormente analisado para identificar de novos biomarcadores de risco neoplásico.

2. **Sangue:**

A realização de Ecoendoscopia é feita sob sedação, sendo necessário puncionar uma veia do braço, pois o sedativo é administrado pela veia (via endo-venosa). Aquando da punção da veia necessária à sedação será realizada uma colheira de 15 ml de sangue que será armazenamento para análise posterior. Serão analisados os biomarcadores de risco neoplásico no quisto do pâncreas e também em sangue periférico.

Compensações e Pagamentos

Não haverá qualquer compensação financeira por participar neste estudo.

Confidencialidade dos registos

Como parte do estudo, a informação médica sobre o seu estado clínico e as características dos quistos será registada, analisada e reportada de forma anónima. Os dados pessoais recolhidos são os estritamente necessários para cumprir com os objetivos do estudo e serão processados pelos elementos da equipa de investigação, exclusivamente para a finalidade descrita. Os resultados deste estudo poderão ser apresentados em reuniões ou publicações, no entanto, a identidade dos participantes não será revelada.

CONSENTIMENTO INFORMADO

Eu, _____, com o número do IPOLFG _____, concordo em participar no estudo "REGISTO DE QUISTOS DO PÂNCREAS DO IPOLFG".

1. Fui informado(a) de que, na sequência da Ecoendoscopia com punção para avaliação de quisto do pâncreas vão ser armazenadas amostras de líquido quístico remanescente após a análise standard e sangue periférico, sem que isso comprometa o diagnóstico final ou implique qualquer procedimento invasivo adicional.

2. Concordo que o material seja armazenado e analisado no IPOLFG ou em laboratório de investigação associado ao IPOLFG.

3. Autorizo que, no contexto do estudo, os investigadores possam aceder a dados do meu processo clínico e que registem os dados dos exames de imagem que irei realizar futuramente.

4. Fui informado de que os resultados do estudo estão sujeitos a sigilo médico e que os doentes permanecerão anónimos.

5. Fui informado de que posso desistir do estudo a qualquer momento, sem que o meu seguimento clínico no IPOLFG seja afetado.

Participante: _____

Lisboa, ___/___/____ (data escrita pelo doente)

Confirmo que expliquei pessoalmente ao indivíduo acima identificado o objetivo e a metodologia do estudo, assim como os procedimentos propostos e suas implicações.

Médico: _____ Cédula Profissional: _____

Lisboa, ___/___/____ (data escrita pelo doente)

NÚMERO DO DOENTE_____

3. Ethical Committee Approvals

IPOLFG, EPE	Apreciação e Votação de Parecer	CE
	INSTITUTO PORTUGUÊS DE ONCOLOGIA DE LISBOA FRANCISCO GENTIL, EPE Comissão de Ética	

Apreciação do Parecer

Data da Reunião:04-01-2018

Título do Projeto: " Biomarkers for classification and Risk Assessment of pancreatic cystic neoplasms" - UIC/1143

A Comissão de Ética para a Saúde (CES) do Instituto Português de Oncologia de Lisboa Francisco Gentil, EPE, em reunião realizada nesta data, apreciou a fundamentação do perito sobre o pedido para realização de projeto de investigação acima identificado.

O processo foi votado pelos membros da CES presentes:
Presidente – Dra. Filomena Pereira
Doutor Adelino Cardoso, Dra Cristina Nave, Dra Manuela Paiva, Enfermeira Maria Manuel Pinto;

Resultado da Votação:

Parecer: Parecer Favorável (fundamentação em anexo)

Data: 04-01-2018

A Presidente da Comissão de Ética para a Saúde
do IPOLFG-EPE

Dra Filomena Pereira

CE.MOD.101.1 1/1

Parecer da Comissão de Ética sobre o Projecto de Investigação intitulado: "Biomarkers for Classification and Risk Assessment of Pancreatic Cystic Neoplasms "- UIC/1143.

O presente projecto de investigação é proposto pela Drª Sandra Faias, médica gastrenterologista no IPOLFG, E.P.E., e insere-se no âmbito do programa do seu doutoramento. Tem como objectivo caracterizar eventos genéticos e epigenéticos em quistos do pâncreas puncionados por ecoendoscopia, de forma a identificar biomarcadores que permitam classificar e estratificar o risco de malignidade dos quistos pancreáticos, o que irá permitir uma intervenção mais precoce nas lesões pré malignas e evitar a progressão da doença. Trata-se assim de um estudo de grande pertinência que contribuirá para uma maior acuidade no diagnóstico, possibilitando o tratamento das lesões precursoras do adenocarcinoma do pâncreas e melhorando o prognóstico da doença.

Em termos metodológicos prevê a realização de 2 estudos: um estudo retrospectivo de uma base de dados de doentes submetidos a ecoendoscopia com punção de quistos do pâncreas entre 2006 e 2016, todos com liquido quistico remanescente congelado, depois da avaliação laboratorial standard, o que permite efetuar as análises mutacionais por sequenciação pretendidas e prevê também a realização de um estudo prospetivo observacional de doentes submetidos a ecoendoscopia com punção de quistos do pâncreas, e em seguimento no IPOLFG.

Para o estudo retrospectivo na há qualquer intervenção adicional para os doentes, apenas execução experimental no liquido quistico sobrante e que foi previamente colhido na pratica clinica corrente.

Para o estudo prospectivo para além da ecoendoscopia com punção do quisto pancreático necessária à avaliação diagnóstica, será necessário a colheita de 15 ml de sangue que será efectuada aquando da punção da veia periférica para a sedação necessária para a realização do procedimento endoscópico. Todos os participantes darão o seu consentimento informado para a participação no estudo o qual contem, em nosso entender, informação clara e adequada sobre os objectivos do estudo e do que envolve a participação, encontrando-se também salvaguardados os aspetos da natureza voluntária da participação, da possibilidade de desistência em qualquer altura sem que isso interfira na assistência clínica prestada, da confidencialidade e do anonimato dos dados recolhidos.

O estudo tem o parecer favorável do Diretor do Serviço onde se irá realizar o estudo e encontra-se referido que a sua concretização envolve uma despesa de 35.500,00 €, encontrando-se descrito pela investigadora que não será suportada pelo IPOLFG e

que irá concorrer a bolsa da LPCC e da Sociedade Portuguesa de Gastrenterologia, pelo que importa esclarecer este aspeto.

Face ao exposto, entende esta Comissão que no que se refere às questões éticas de principio, as mesmas encontram-se salvaguardadas, pelo que o parecer ético é favorável à realização do presente estudo.

Lisboa, 4 de Janeiro de 2018

Pela Comissão de Ética

Maria Manuel Pinto

COMISSÃO DE ÉTICA

IPOLFG, EPE	Apreciação e Votação de Parecer INSTITUTO PORTUGUÊS DE ONCOLOGIA DE LISBOA FRANCISCO GENTIL, EPE Comissão de Ética	CE

Apreciação do Parecer

Data da Reunião: 08-11-2018

Título do Projeto: "Chromogranin a levels in Diagnosis of pancreatic cystuc Neoplasms" - UIC/1224

A Presidente da Comissão de Ética para a Saúde (CES) do Instituto Português de Oncologia de Lisboa Francisco Gentil, EPE, apreciou a fundamentação do perito sobre o pedido de realização do projeto acima identificado.

Resultado da Votação:

Parecer: Favorável (fundamentação em anexo)

Data: 08-11-2018

A Presidente da Comissão de Ética para a Saúde
do IPOLFG-EPE

Dra Filomena Pereira

CE.MOD.101.1 1/1

Parecer da Comissão de Ética Para a Saúde Sobre o Projecto de Investigação **"Chromogranin a Levels in Diagnosis of Pancreatic Cystic Neoplasms" - UIC/1224.**

O estudo supra referido pretende estudar o papel do nível de CgA no líquido quístico pancreático, enquanto possível marcador diferencial bioquímico dos tumores neuroendócrinos pancreáticos quísticos.

Na Instituição, os serviços envolvidos respeitam aos Serviços de Gastrenterologia, Endocrinologia e Anatomia Patológica e a responsável pela investigação é a Dra. Sandra Faias do Serviço de Gastrenterologia.

Trata-se de um estudo observacional, laboratorial e retrospectivo. Em concreto, as amostras utilizadas no estudo correspondem a excedente de líquido quístico obtido por ecoendoscopia com aspiração por agulha fina (a qual permite a análise detalhada de imagens e líquido quístico para análises citológicas e bioquímicas), armazenado, após avaliação clínica *standard*, com CEA e citologia no líquido quístico. Prevê-se a inclusão de 16 doentes (relativos ao período compreendido entre 2008 e 2018).

Não surge no protocolo apresentado referência à confidencialidade, anonimato e consentimento informado. Entende esta Comissão, sublinhar que de acordo com o preconizado nas boas práticas de actuação em investigação, a dispensa de consentimento informado não se prende unicamente com a ausência de informação pessoal que ponha em causa a identidade da pessoa, nem directamente com o facto de ser um estudo retrospectivo. Com efeito, o consentimento é requerido não só para situações de participação directa nas investigações, mas também para que seja viável o acesso aos dados de saúde e pressupõe a pré-existência de um tratamento de dados pessoais previamente autorizado. Todavia, o entendimento desta Comissão é favorável à dispensa do consentimento informado, dado que o mesmo provavelmente inviabilizaria a concretização da investigação, a qual se reveste de interesse científico e potencial melhoria dos cuidados prestados. Sublinha-se que os dados deverão ser anonimizados, agrupados e utilizados apenas para fins estatísticos, não se procedendo à identificação do doente.

O parecer da Comissão de Ética Para a Saúde do Instituto Português de Oncologia de Lisboa Francisco Gentil, E.P.E. é favorável à realização desta investigação, uma vez que não constituiu risco para os participantes e pode ajudar a optimizar os cuidados de saúde.

Lisboa, 08 de Novembro 2018

P' la Comissão de Ética

Ana Cristina Nave

(Ana Cristina Nave)

COMISSÃO DE ÉTICA

IPOLFG, EPE	Apreciação e Votação de Parecer INSTITUTO PORTUGUÊS DE ONCOLOGIA DE LISBOA FRANCISCO GENTIL, EPE Comissão de Ética	CE

Apreciação do Parecer

Data da Reunião: 08-11-2018

Título do Projeto: "Glucose levels for diagnosis of pacreantic cystic neoplasms" - UIC/1225

A Presidente da Comissão de Ética para a Saúde (CES) do Instituto Português de Oncologia de Lisboa Francisco Gentil, EPE, apreciou a fundamentação do perito sobre o pedido de realização do projeto acima identificado.

Resultado da Votação:

Parecer: Favorável (fundamentação em anexo)

Data: 08-11-2018

A Presidente da Comissão de Ética para a Saúde
do IPOLFG-EPE

Dra Filomena Pereira

CE.MOD.101.1 1/1

Parecer da Comissão de Ética Para a Saúde Sobre o Projecto de Investigação **"Glucose Levels for Diagnosis of Pancreatic Cystic Neoplasms" - UIC/1225.**

O estudo supra referido pretende avaliar o nível de glicose em líquido quístico, isolado e em conjunto com o CEA e a citologia, no diagnóstico de neoplasias quísticas pancreáticas mucinosas.

Na Instituição, os serviços envolvidos respeitam aos Serviços de Gastrenterologia e Anatomia Patológica e a responsável pela investigação é a Dra. Sandra Faias do Serviço de Gastrenterologia.

Trata-se de um estudo observacional, laboratorial e retrospectivo. Em concreto, as amostras utilizadas no estudo correspondem a excedente de líquido quístico obtido por ecoendoscopia com aspiração por agulha fina (a qual permite a análise detalhada de imagens e líquido quístico para análises citológicas e bioquímicas), armazenado, após avaliação clínica *standard*, com CEA e citologia no líquido quístico. Prevê-se a inclusão de 20 doentes (relativos ao período compreendido entre 2008 e 2018).

Não surge no protocolo apresentado referência à confidencialidade, anonimato e consentimento informado. Entende esta Comissão, sublinhar que de acordo com o preconizado nas boas práticas de actuação em investigação, a dispensa de consentimento informado não se prende unicamente com a ausência de informação pessoal que ponha em causa a identidade da pessoa, nem directamente com o facto de ser um estudo retrospectivo. Com efeito, o consentimento é requerido não só para situações de participação directa nas investigações, mas também para que seja viável o acesso aos dados de saúde e pressupõe a pré-existência de um tratamento de dados pessoais previamente autorizado. Todavia, o entendimento desta Comissão é favorável à dispensa do consentimento informado, dado que o mesmo provavelmente inviabilizaria a concretização da investigação, a qual se reveste de interesse científico e potencial melhoria dos cuidados prestados. Sublinha-se que os dados deverão ser anonimizados, agrupados e utilizados apenas para fins estatísticos, não se procedendo à identificação do doente.

O parecer da Comissão de Ética Para a Saúde do Instituto Português de Oncologia de Lisboa Francisco Gentil, E.P.E. é favorável à realização desta investigação, uma vez que não constituiu risco para os participantes e pode ajudar a optimizar os cuidados de saúde.

Lisboa, 08 de Novembro 2018

P' la Comissão de Ética

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COMISSÃO DE ÉTICA

4. Publications

REVIEW

KRAS in Cyst Fluid Obtained by Endoscopic Ultrasound–Fine-Needle Aspiration in Pancreatic Cystic Lesions

A Systematic Review and Meta-analysis

Sandra Faias, MD,*†‡ Luisa Pereira, PhD,‡§ Ângelo Luís, PhD,‡|| Marília Cravo, MD, PhD,¶# António Dias Pereira, MD, PhD,* and Joana Torres, MD, PhD¶

Abstract: To evaluate the diagnostic accuracy of *KRAS* mutation in pancreatic cystic fluid and compare it with carcinoembryonic antigen and cytology, we identified studies with cyst fluid obtained by endoscopic ultrasound prior to surgery. We classified cysts as malignant, premalignant, and benign. A random-effects model was used for quantitative meta-analysis. Pooled sensitivities, specificities, and summary receiver operating characteristic curve analysis were conducted. We analyzed 16 studies, with 3429 patients, including 731 referred for surgery. Carcinoembryonic antigen was better for clinically significant cysts (premalignant and malignant) with sensitivity = 0.58 (95% confidence interval [CI], 0.53–0.65), specificity = 0.9 (95% CI, 0.76–0.97), and area under the curve (AUC) = 0.69. Cytology performed better in malignant cysts, with sensitivity = 0.37 (95% CI, 0.27–0.48), specificity = 0.96 (95% CI, 0.93–0.98), and AUC = 0.78. Isolated, *KRAS* mutation failed the diagnosis of malignant and significant cysts, with sensitivities = 0.43 (95% CI, 0.34–0.43) and 0.46 (95% CI, 0.42–0.51), specificities = 0.62 (95% CI, 0.56–0.68) and 0.97 (95% CI, 0.92–0.99), and AUCs = 0.56 and 0.53, respectively. Carcinoembryonic antigen and cytology are more accurate than *KRAS*. Additional studies are lacking to recommend *KRAS* as a single diagnostic test.

Key Words: CEA, cytology, EUS, EUS-FNA, *KRAS*, pancreatic cyst

(*Pancreas* 2019;48: 749–758)

Pancreatic cystic neoplasms (PCNs) are increasingly found in clinical practice, due to an aging population and the routine use of high-quality abdominal imaging.[1] The importance of PCNs is related to malignant potential, high frequency, and significant morbidity and mortality of surgical treatment. Therefore, there is an urgent need to find noninvasive and reliable markers of malignant and high-risk premalignant PCNs.

In clinical practice, after clinical and imaging findings of a potentially significant lesion, including mucinous premalignant or malignant cysts, endoscopic ultrasound with fine-needle aspiration (EUS-FNA) for cystic fluid analysis for carcinoembryonic antigen (CEA) and cytology became standard in decision making. Carcinoembryonic antigen is the most accurate for diagnosing mucinous cysts, which are premalignant lesions, whereas cytology is highly specific for malignancy diagnosis.[2] Treatment options, including surgery, follow-up, or no additional evaluation, rely on imaging and pancreatic cystic fluid (PCF) analysis, but a significant part remains indeterminate, with approximately one-third of preoperative diagnosis being incorrect.[3,4]

In this clinical context, pancreatic cyst fluid analysis for molecular markers has shown that *KRAS* mutations may be specific for mucinous cysts[5–7] and that simultaneous *KRAS/GNAS* mutations are specific of intraductal papillary mucinous neoplasms (IPMNs).[8,9] Currently, next-generation sequencing (NGS), a very sensitive technique for detection of genetic mutations, can be considered in indeterminate PCNs or if it modifies patient management.[10] Numerous studies have shown that DNA molecular analysis of aspirates obtained by EUS-FNA provides a better characterization of PCNs compared with current methods used in clinics.[11–19] However, these studies have generally included a limited number of patients, and results are not consistent among studies. Currently, the integration of molecular analysis in routine clinical practice is still a matter of debate.

We therefore performed a systematic review and meta-analysis of all previous studies with *KRAS* mutational analysis performed by NGS in PCF obtained preoperatively by EUS-FNA. All samples with a surgical pathology as reference standard for diagnosis were evaluated. Our aim was to investigate the accuracy of *KRAS* mutational analysis for diagnosis of mucinous and significant (mucinous and malignant) PCNs and compare it with routine standard diagnosis, with CEA and cytology.

MATERIALS AND METHODS

Search Strategy and Eligibility Criteria

The systematic review and meta-analysis reported here were conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines,[20] and the protocol was registered at PROSPERO (CRD42018097268). A comprehensive search of databases, including MEDLINE, Scopus, Web of Science, and SCIELO, for the past 18 years (January 1, 2000, to March 31, 2018) and restricted to human studies was performed. No language restrictions were applied. The following search terms were used: "pancreas," "cyst," "molecular," and "analysis." Additional search of related articles and

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All authors have contributed to the study concept and design. S.F., L.P., and A.L. acquired and interpreted the data and drafted the manuscript, under the supervision of all senior authors. M.C., A.D.P., and J.T. critically revised the manuscript, and all authors approved the final version of the manuscript.
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hand search of references of all selected studies were performed, adding additional publications.

Inclusion Criteria

Published studies were included in the meta-analysis if they (1) analyzed mutational analysis of *KRAS* using highly sensitive techniques, such as NGS; (2) analyzed a cohort of patients with pancreatic cysts and symptomatic or incidental findings; (3) cysts were evaluated by EUS-FNA with PCF analysis; and (4) all patients had a definitive diagnosis with a surgically resected specimen.

Exclusion Criteria

Exclusion criteria were as follows: (1) studies on molecular markers other than *KRAS* mutation; (2) studies involving solid pancreatic lesions; (3) studies performed in PCF not obtained by EUS-FNA; (4) studies with cytology and clinical information as standard criterion of diagnosis without a surgical pathology specimen as reference standard; and (5) reviews, case reports, letters to editor, exploratory studies, and articles published only in abstract form.

Two reviewers (S.F. and A.L.) independently judged study eligibility, and disagreements were resolved by consensus.

Histological Criteria and Tests Under Investigation

Based on the World Health Organization tumor classification, PCN diagnosis were reviewed and classified into 1 of 3 groups: (1) malignant cysts (adenocarcinoma or high-grade dysplasia in IPMNs and mucinous cystic neoplasms, secondary cystic adenocarcinomas, and cystic pancreatic neuroendocrine tumors); (2) premalignant mucinous cysts (IPMNs and mucinous cystic neoplasms with low- or intermediate-grade dysplasia); and (3) benign cysts (serous cystadenomas, pseudocysts, and other benign cysts).

The index test was molecular analysis with *KRAS* mutation, because it is the most frequent mutation. The comparators were as follows: (1) CEA (cutoff value >192 ng/mL) for diagnosis of mucinous cystic lesions and (2) cytology that was considered positive if samples were read as atypical, suspicious, positive, or malignant. Cytology was considered negative if samples were read as indeterminate, acellular, or negative for malignancy. It should be noted that a diagnosis of atypia in a cytological evaluation does not warrant a malignancy diagnosis requiring surgery.

Outcomes

The primary outcome was to assess the diagnostic accuracy of *KRAS* mutation in PCF for diagnosis of malignant and significant PCNs. The secondary outcome was to compare the accuracy of *KRAS* mutation with current standard of diagnosis, with PCF analysis for CEA and cytology, in malignant and significant PCNs.

Data Extraction and Quality Assessment

Selected articles' data were extracted independently by 2 reviewers (S.F. and A.L.), who were blinded to publication details, onto a predefined worksheet. Disagreements were discussed and reviewed by a third reviewer (L.P.).

Data extraction included the name of first author, publication year, study design (prospective, cross-sectional, retrospective), sample size (all patients included in the study), number of patients referred for surgery (surgical cohort), number of malignant lesions, distribution of cyst types (malignant, premalignant, benign), number of patients with a CEA of greater than 192 ng/mL, a positive cytology, and *KRAS* mutation detection.

Methodological quality of primary studies included was assessed by 2 authors (S.F. and A.L.) using the modified QUADAS-2 (Quality Assessment Tool for Diagnostic Accuracy Studies version 2) tool,[21] which evaluates the quality of articles for systematic reviews of diagnostic accuracy studies in 4 domains, including patient selection, index test, reference standard, and flow and timing, for risk of bias and applicability concerns.

Statistical Analysis and Data Synthesis

Our reference standard was surgical specimen that classified PCNs into 3 groups: malignant, premalignant, and benign cysts. This resulted in a 2×3 table: positive or negative test result in each of the 3 groups, for each of the 3 tests, *KRAS* (index test), cytology, and CEA (comparator tests).

To calculate test accuracy and to reflect the categories that are used in clinical practice and guide management, we constructed 2×2 tables, to evaluate the ability of the index test and comparator tests to discriminate malignant from nonmalignant (all cysts except those proven to be malignant) and significant (proven malignant and premalignant cysts) from nonsignificant cysts (proven benign cysts).

The data of the 2×2 tables were used to calculate sensitivity and specificity for each study. We present individual study results graphically by plotting the estimates of sensitivity and specificity (and their 95% confidence intervals [CIs]) in both forest plots and on the summary receiver operating characteristic (sROC) curve plots. The area under the curve (AUC) is equal to the probability that if a pair of relevant and nonrelevant cysts is selected at random, the relevant cyst will have a higher or positive test result than the nonrelevant cyst. Pooled estimates of the sensitivity and specificity were obtained by DerSimonian-Laird method (random-effects model) to incorporate variation among studies, when data are heterogeneous.

Heterogeneity was investigated in the first instance through visual examination of forest plots of sensitivities and specificities and through visual examination of the ROC plot of the raw data. Last, we used the χ^2 test to evaluate if the differences across the studies were greater than expected by chance alone. A low P value suggested presence of heterogeneity. In addition, we used the statistic I^2 of Higgins that allowed us to quantify the amount of heterogeneity.[22,23] The scale of I^2 has a range of 0% to 100% and values of 25%, 50%, and 75% are considered low, moderate, and high heterogeneity, respectively.

Publication Bias

To analyze the publication bias in meta-analyses of sensitivity and specificity, we used Deeks' test. This test, developed for diagnostic test accuracy (DTA), is the least biased and is recommended in the *Cochrane Handbook for Systematic Reviews of Diagnostic Test Accuracy*.[24,25]

We used Meta-DiSc (version 1.4; Meta-analysis of Diagnostic and Screening Tests)[26] for assessment of diagnostic yield of the studies and SPSS Statistics (version 23; IBM Corp, Armonk, NY) for Deeks' test.

RESULTS

Search Results and Characteristics of the Studies Included

Our search found 496 study titles and abstracts. Figure 1 describes the selection process of the articles included in this study.

After abstract screening and full-text review, 16 studies met the inclusion criteria and were considered suitable for qualitative

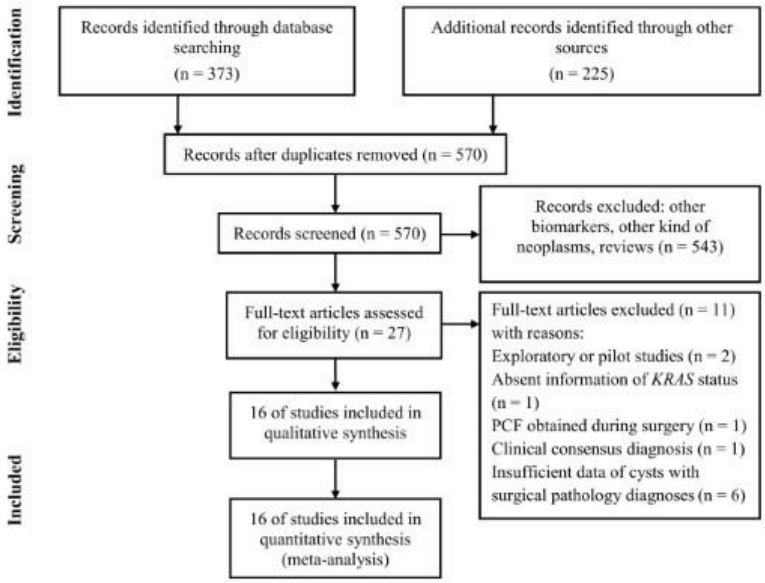


FIGURE 1. Flowchart of literature search according to PRISMA flow diagram summarizing study selection.

and quantitative analyses. The design was retrospective in 13 and prospective in 3 articles, with 3 studies published from years 2005 to 2009, 8 from years 2010 to 2014, and 5 from years 2015 to 2018.

These 16 studies included a total of 3429 patients, of which 731 (21%) underwent surgical resection and had a surgical pathology specimen available as reference standard and were included for analysis. Patients of studies in which data were available for the overall series (aggregating results of surgical and clinical surveillance cohorts) but were not discretely available for the surgical cohort were excluded from analysis.

The characteristics of the studies, surgical pathology diagnoses, and cystic fluid analysis details are in Table 1.

Quality Assessment

Results of methodological quality of the primary studies included are presented in Figure 2, which was sketched with templates available at www.quadas.org. All studies included in this review showed a "low-risk" classification, as the index test (*KRAS* mutation analysis) and the reference standard (surgical pathology specimen) were reliable and mentioned in all studies. However, a "high risk" of selection bias was demonstrated in a patient and in flow and timing because only a small proportion of the patients evaluated in all studies, except 1, were included in the analysis. In fact, many patients were excluded in all studies as the inclusion criterion requiring surgical pathology as diagnostic reference was not met. Applicability concerns regarding patient selection were also significant in all studies, because the subgroup of PCNs referred for surgery is more often malignant than for patients with pancreatic cysts on clinical surveillance, which would also be targeted with this review.

KRAS Mutation

Fourteen articles were included in the meta-analysis for diagnostic accuracy of *KRAS* mutation. For each of the 2 definitions of relevant cyst, forest plots of sensitivity and specificity with heterogeneity denoted are shown in Figure 3.

The definitions of malignant and significant cysts resulted in a different range of specificity and sensitivity of the studies included. In the first case, both sensitivity and specificity varied from 0% to 100% and in the second case sensitivity varied from 12% to 100% and specificity from 50% to 100%. In the first subgroup, the wide range of sensitivity was largely due to chance variation because of small numbers of patients with the target condition (proven malignant cysts) in the different studies (median, 6; range, 1–31). For instance, if there was only 1 patient with a proven malignant cyst in a study, and this patient had a positive test, the sensitivity would be 100%, but if he/she had a negative test result, the sensitivity would be 0%. Small numbers of patients with nonmalignant cysts in some studies (median, 10; range, 1–111) also led to a wide range of specificity.

For each of the 2 subgroups, there occurred a moderate heterogeneity in sensitivity ($I^2 = 46.8\%$ vs $I^2 = 65.0\%$) and specificity ($I^2 = 52.5\%$ vs $I^2 = 34.3\%$), and therefore random-effects models were used. In malignant cysts, the pooled sensitivity was 0.43 (95% CI, 0.34–0.53), and the pooled specificity was 0.62 (95% CI, 0.56–0.68). In significant cysts, the sensitivity was 0.46 (95% CI, 0.42–0.51) with a specificity of 0.97 (95% CI, 0.92–0.99).

Figure 4 displays the sROC curves of *KRAS* analysis, showing the sensitivity of the individual articles mapped on the vertical scale, 1-specificity on the horizontal scale, and summary (sensitivity, 1-specificity) point marked, as well as the sROC curve and the confidence region for the summary (sensitivity, 1-specificity) points. The area under the sROC curve ± SE was 0.5551 ± 0.0659 in malignant cysts and 0.5290 ± 0.1424 in significant cysts. The results of the studies had greater variation in malignant cysts, as shown by the wide confidence region.

The median prevalence of malignant cysts and significant cysts was 29.6% and 82.5%, respectively (range, 9.1%–85.7% and 50%–100%, respectively). This prevalence was based on the proportion of proven malignant and proven significant cysts in the studies.

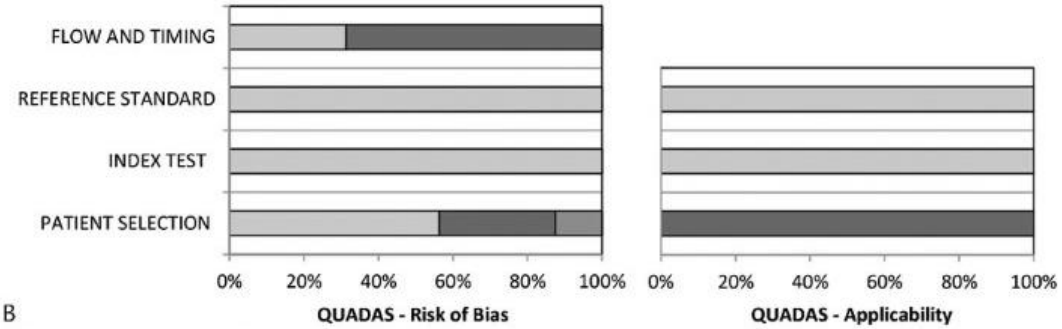
TABLE 1. Characteristics of Studies and Test Results Included in the Analysis

Author, Year	Prospective/ Retrospective (Study Period)	Diagnosis	Sample Size*	Surgical Cohort	Malignant Cysts (Surgical Pathology)	Mucinous Premalignant Cysts (Surgical Pathology)	Benign Cysts (Surgical Pathology)	CEA >192 ng/mL	Positive Cytology	*KRAS* Mutation	CEA ≥192 in Malignant/ Mucinous/ Benign	Cytology+ in Malignant/ Mucinous/ Benign	*KRAS* Mutation in Malignant/ Mucinous/ Benign
Schoedel et al,[12] 2006	Retrospective (NA)	Pathology	16	16	4	12	0	NA	2	4	NA	2/0/0	2/2/0
Sreenarasimhaiah et al,[13] 2009	Retrospective (Jul 2006–Nov 2007)	Pathology	60; 20 study cohort	6	6	0	0	4	0	2	4/0/0	0/0/0	2/0/0
Sawhney et al,[15] 2008	Retrospective (2006–2007)	Pathology or cytology	111; 100 study cohort	19	5	12	2	14	1	2	A/B/0 A + B = 14	1/0/0	1/1/0
Mertz,[19] 2011	Retrospective (May 2007–March 2008)	Pathology or cytology	60 study cohort	10	0	7	3	NA	5	4	NA	0/4/1	0/3/1
Toll et al,[27] 2010	Retrospective (2007–2010)	Pathology or cytology	63	2	1	0	1	NA	1	1	NA	1/0/0	1/0/0
Panarelli et al,[28] 2012	Prospective (2005–2010)	Pathology	18†	4	1	3	0	1	1	1	0/1/0	1/0/0	0/1/0
Rockacy et al,[29] 2013	Retrospective (NA)	Pathology or cytology	134	51	10	18	16	NA	10	NA	NA	10/0/0	10/NA/NA
Nikiforova et al,[30] 2013	Retrospective (Nov 2006–Oct 2012)	Pathology or cytology	603	142	31	85	26	NA	NA	53	NA	NA	7/46/0
Al-Haddad et al,[31] 2014	Prospective (2008–2012)	Pathology or cytology	286	48	6 + 4	32	6	24‡	7	16‡	A/B/0 A + B = 24	NA/NA/NA	C/D/NA C + D = 16
Singhi et al,[9] 2014	Retrospective (2006–2013)	Pathology or cytology	91	83	19	57	7	36	NA	36	36	NA	8/35/0§
Kung et al,[32] 2014	Retrospective (2010–2013)	Pathology or cytology	72	6	5	0	1	4	2	2	4/0/NA	2/0/0	2/0/NA
Winner et al,[33] 2015	Retrospective (2006–2012)	Pathology	200	40	10 + 3	23	4	19	3	16	A/B/1 A + B = 18	2/A/B A + B = 1	4/A/B A + B = 12
Jones et al,[34] 2016	Prospective (Mar 2013–Feb 2014)	Pathology or cytology	86	10	6	4	0	5	3	8	3/2/0	3/0/0	4/4/0
Singhi et al,[35] 2016	Retrospective (Jan 2014–May 2015)	Pathology or clinicopathologic	225	41	13	13	15	NA	6	17	NA	5/0/1	10/7/0
Kadayifci et al,[5] 2016	Retrospective (2006–2014)	Pathology or clinicopathologic	943	147	25 + 12	83	27	72	NA	50	A/B/2 A + B = 70	NA	C/D/0 C + D = 50
Singhi et al,[7] 2018	Prospective (Jan 2014–Jul 2017)	Pathology or cytology	595	102	19 + 9	47	27	NA	7	47	NA	7/0/0	16/31/0

*All patients in the study.
†Twenty cysts in 18 patients.
‡In 38 premalignant mucinous cysts.
§*GNAS* or *KRAS* mutation.
Atypical cytology was grouped as benign in this study.
NA indicates nonavailable data.

Study	RISK OF BIAS				APPLICABILITY CONCERNS		
	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD	FLOW AND TIMING	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD
Schoedel et al, 2006[12]	☺	☺	☺	☺	☹	☺	☺
Sreenarasimhaiah et al, 2009[13]	☺	☺	☺	☹	☹	☺	☺
Sawhney et al, 2009[15]	☺	☺	☺	☹	☹	☺	☺
Mertz et al, 2011[19]	☺	☺	☺	☹	☹	☺	☺
Toll et al, 2010[27]	☺	☺	☺	☹	☹	☺	☺
Panarelli et al, 2012[28]	?	☺	☺	☹	☹	☺	☺
Rockacy et al, 2013[29]	?	☺	☺	☹	☹	☺	☺
Nikiforova et al, 2013[30]	☹	☺	☺	☹	☹	☺	☺
Al-Haddad et al, 2014[31]	☺	☺	☺	☹	☹	☺	☺
Singhi et al, 2014[5]	☹	☺	☺	☹	☹	☺	☺
Kung et al, 2014[32]	☹	☺	☺	☹	☹	☺	☺
Winner et al, 2015[33]	☹	☺	☺	☹	☹	☺	☺
Jones et al, 2015[34]	☺	☺	☺	☹	☹	☺	☺
Singhi et al, 2016[35]	☺	☺	☺	☹	☹	☺	☺
Kadayifci et al, 2016[5]	☺	☺	☺	☺	☹	☺	☺
Singhi et al, 2017[7]	☺	☺	☺	☺	☹	☺	☺

A ☺Low Risk, ☹High Risk, ? Unclear Risk



B

FIGURE 2. Quality assessment of the studies included using QUADAS-2. A, Tabular presentation of risk bias for each study. B, Graphical display of bias.

Cytology

Twelve articles were included in the meta-analysis for diagnostic accuracy of cytology. Figure 5 shows the forest plots of sensitivity and specificity for the 2 defined subgroups of cysts. The forest plots for cytology show variable sensitivities within the articles, from 0 to 1, which can be due to the small numbers of patients with the target condition in some studies.

In the malignant and significant cysts groups, respectively, there were 6 and 2 studies, respectively, that recorded sensitivity of cytology as scoring greater than or equal to 0.5.

For each of the 2 subgroups, there existed heterogeneity in sensitivity (I^2 = 69.2% vs I^2 = 60.4%) and specificity (I^2 = 64.9% vs I^2 = 27.7%), and therefore random-effects models were used. In malignant cysts, the pooled sensitivity was 0.37 (95% CI, 0.27–0.48), and the pooled specificity was 0.96 (95% CI, 0.93–0.98). In significant cysts, the sensitivity was 0.19 (95% CI, 0.13–0.25) with a specificity of 0.94 (95% CI, 0.86–0.98).

The results were plotted as a symmetrical sROC curve (Fig. 4). The area under the sROC curve ± SE was 0.7788 ± 0.1309 in malignant and 0.4805 ± 0.1542 in significant cysts.

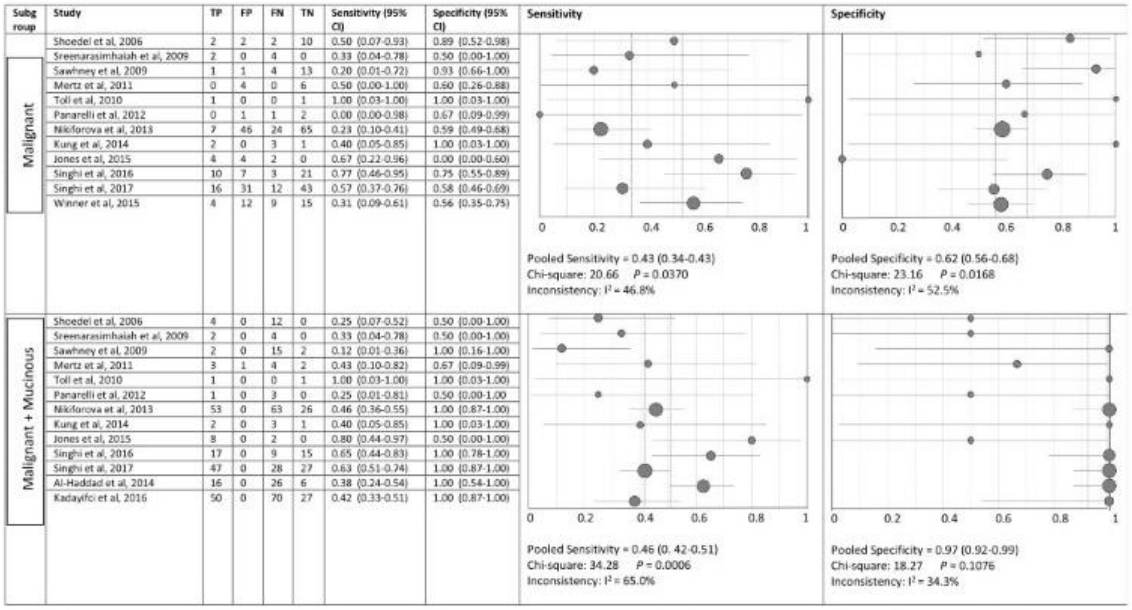


FIGURE 3. Forest plots of the included studies for *KRAS*. Between brackets are the 95% CIs of the sensitivity and specificity. The figure shows the estimated sensitivity of the study (circle) and its 95% CI (horizontal line). The area of the circle reflects the weight that the study contributes to the meta-analysis.

The median prevalence of malignant and significant cysts was 29.4% and 86.4%, respectively (range, 9.1%–85.7% and 50%–100%, respectively).

CEA Greater Than 192 ng/mL

Eight articles were included in the meta-analysis for diagnostic accuracy of CEA. Because only 4 articles (with few patients) allowed the evaluation of accuracy of CEA greater than 192 ng/mL for diagnosis of malignant cysts, we restricted the analysis to significant cysts. Figure 6 shows the forest plots of sensitivity and specificity. The forest plots for cytology showed a sensitivity from 0.5 to 0.82 and a specificity from 0.5 to 1.

There existed homogeneity in sensitivity (I^2 = 21.2%) and specificity (I^2 = 0%). The pooled sensitivity was 0.58 (95% CI, 0.52–0.65), and the pooled specificity was 0.90 (95% CI, 0.76–0.97).

The area under the sROC curve ± SE was 0.6903 ± 0.1228.

The median prevalence of significant cysts was 89.7% (range, 81.6%–100%).

Publication Bias

Regression analyses of funnel plots were not statistically significant ($P > 0.05$), suggesting that publication bias was not a major determinant.

DISCUSSION

In this systematic review and meta-analysis, we performed a comparative analysis of the current standard tests in PCF obtained by EUS-FNA (CEA and cytology) and molecular analysis (*KRAS* mutation) in PCNs. The comparative analysis included all studies, evaluating the 3 tests separately.

Our meta-analysis is the largest published and included 731 patients, all with molecular analysis performed by NGS preoperatively, and all patients with a surgical pathology specimen as reference standard for diagnosis. We analyzed these 3 markers, for diagnosis of significant as compared with benign cysts and for diagnosis of malignant versus nonmalignant cysts, because relevant clinical decisions apply to these categories.

The comparative analysis of *KRAS*, cytology, and CEA for cyst diagnosis showed that cytology alone had the highest accuracy (AUC = 0.7788) for the diagnosis of malignant cysts, and CEA, the highest accuracy (AUC = 0.6903) for the diagnosis of significant cysts. *KRAS* mutational analysis had the worst performance for both groups of lesions with AUC = 0.551 for malignant and AUC = 0.46 for significant cysts. The specificity of *KRAS* for diagnosis of significant cysts was high (97%), which makes it useful to diagnose these lesions, but because of low sensitivity (46%), *KRAS* should not be used to exclude the diagnosis, as false-negative (FN) results are common. Similar results for *KRAS* were previously published by Guo et al,[36] who analyzed several molecular tests for improving differential diagnosis of PCNs.

As DNA testing continues to evolve, questions remain about its accuracy, how it influences patient management, and in what order it should be performed to better support clinical decisions. Previous studies[6] have shown that DNA testing combined with clinical features increased correct PCNs diagnosis compared with either one. With the multiple recent advances in biomarkers, particularly DNA-based mutations, molecular genetics will probably prove to be useful in management of PCNs.[37] In a previous meta-analysis, cytology in preoperative diagnosis of PCNs has shown low sensitivity for diagnosis,[38] recommending additional tests to improve diagnosis. Another published meta-analysis evaluating diagnostic accuracy of EUS-FNA with CEA and cytology in differentiating mucinous cysts has demonstrated to be accurate to confirm the diagnosis but performs poorly in excluding it.[39] The role of *KRAS* as individual screening test has also been analyzed before[40] with poor accuracy and added benefit coming from a combined approach with cytology. Finally, a recently published meta-analysis supporting *KRAS*, *GNAS*, and *RNF43* mutations

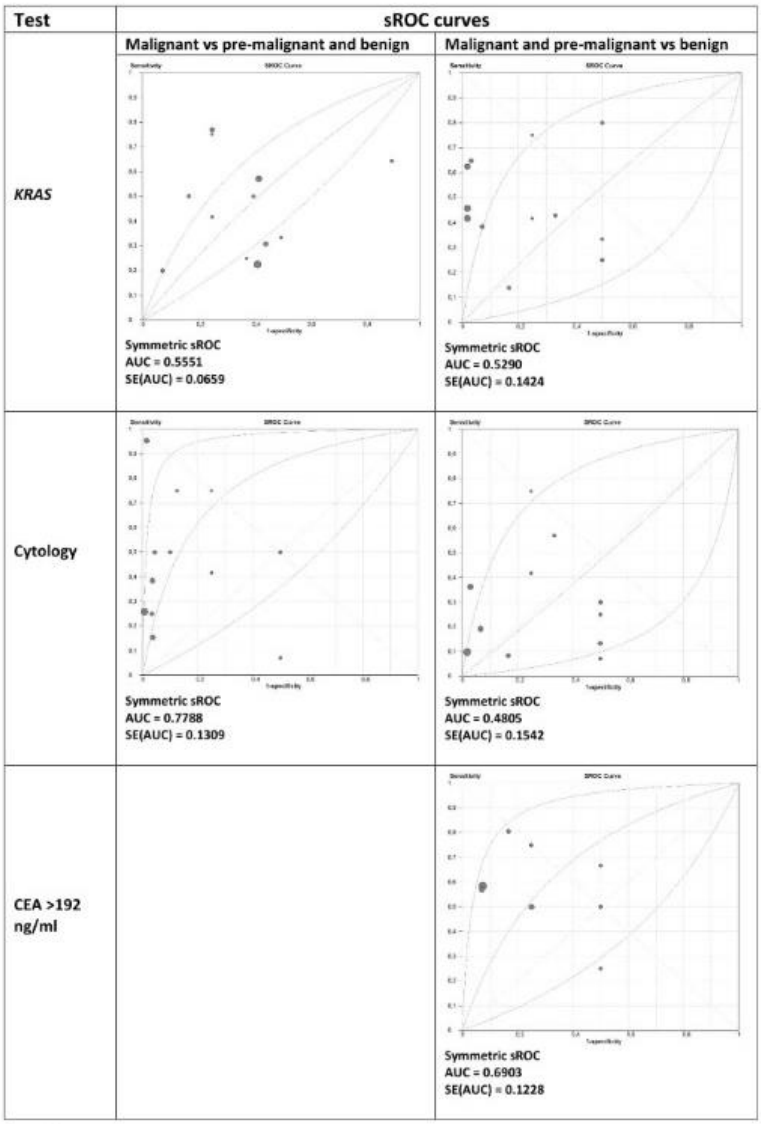


FIGURE 4. Summary ROC plots.

as diagnostic markers of IPMNs[41] used different methods for mutation detection, different tumor materials, and clinicopathologic data as reference standard for diagnosis, which may limit its clinical application in pancreatic cystic lesions, in which mutational analysis is performed solely in cystic fluid.

In our study, the pooled sensitivities of *KRAS*, cytology, and CEA, besides being limited, also varied considerably. On the other hand, specificity was uniformly high for the tests analyzed, particularly for *KRAS* and CEA for diagnosis of significant cysts and cytology for both malignant and significant cysts.

By estimating the pooled sensitivities, we sought to determine which of the tests had a better performance.

For a group of 100 patients with a pancreatic cyst and a prevalence of malignant cysts of 30%, the presence of a *KRAS* mutation would diagnose 13 (true-positive [TP]) and miss 17 (FN), and 27 (false-positive [FP]) would be unnecessarily operated. For a prevalence of significant cysts of 86%, 40 would be correctly diagnosed (TP), 46 would be missed by *KRAS* (FN), and none would be unnecessarily referred for surgery/surveillance (FP).

With respect to cytology, a positive result for the diagnosis of malignant cysts in a group of 100 patients with a prevalence of 30% of malignant cysts would diagnose 11 (TP) and would miss 19 (FN) patients, and 3 (FP) would be unnecessarily referred for surgery. For significant cysts, with a prevalence of 86%, a positive cytology would diagnose 16 (TP) PCNs and would miss 70 (FN) PCNs, and none (FP) would be unnecessarily referred for surgery/surveillance.

If 100 patients with PCNs evaluated with a CEA of greater than 192 ng/mL in PCF and a prevalence of significant cysts of 86%, 52 (TP) would be diagnosed by CEA, and 36 (FN) would

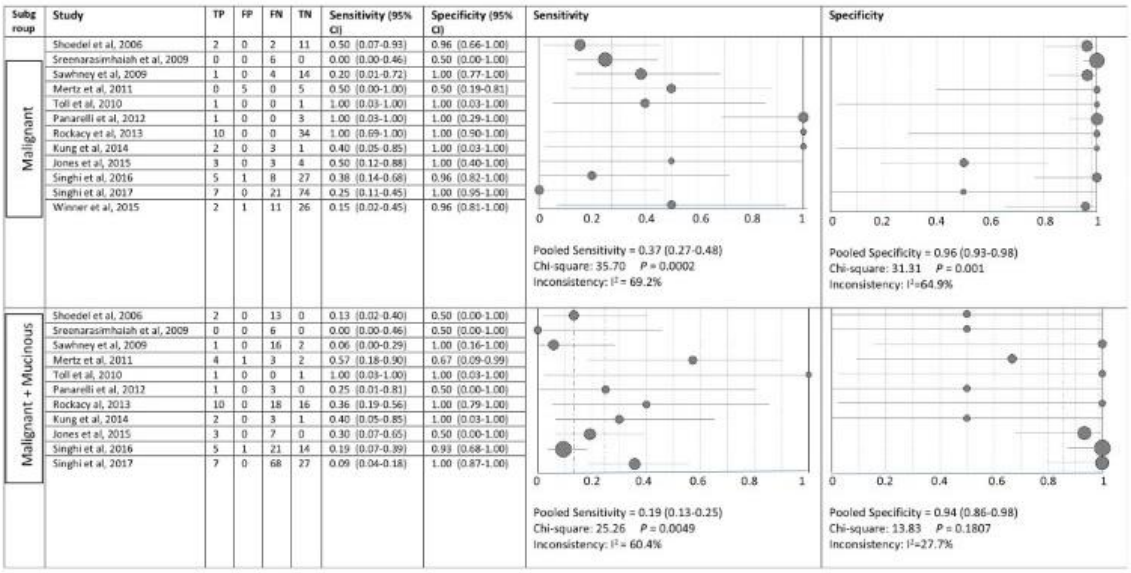


FIGURE 5. Forest plots of the studies included for cytology. In parentheses are the 95% CIs of the sensitivity and specificity. The figure shows the estimated sensitivity of the study (circle) and its 95% CI (horizontal line). The area of the circle reflects the weight that the study contributes to the meta-analysis.

be missed by the test, with 1 (FP) that would be unnecessarily referred for surgery/follow-up.

Although both *KRAS* and CEA are useful for mucinous cyst diagnosis that were classified in this meta-analysis as significant, based on our results we can conclude that CEA would miss fewer PCNs (lower FN rate) with only 1 FP. Concerning malignancy diagnosis, cytology is the best diagnostic test, because although *KRAS* mutation can diagnose more malignant cysts (13 vs 11), it would have significantly higher numbers of FP diagnosis (27 vs 1). We can conclude that *KRAS* mutation is not better than CEA for significant cyst diagnosis and that cytology is the most accurate test for malignancy diagnosis.

However, we should remember that in routine clinical practice a major pitfall for PCNs diagnosis is the frequently scant volume of PCF obtained, precluding routine PCF testing. As mutation analysis requires less volume of PCF, it may be an alternative test in these circumstances. This major advantage of molecular analysis was not possible to evaluate because the volume of cystic fluid obtained was not available in most studies analyzed.

Additionally, combining *KRAS* mutation with conventional testing increased the sensitivity of PCN diagnosis without compromising specificity. We extracted data from the studies analyzed in this meta-analysis to evaluate the added value of *KRAS* in conjunction with cytology and CEA, but the available data were limited to 4 studies[27,28,32,34] (Table 1), making the analysis inconclusive.

The strengths of our work are the use of strict exclusion criteria, with all analyzed patients with an analyzed surgical pathology as the reference standard and avoiding bias related to methodological limitations of the studies evaluated. We chose to include only patients with a surgical pathology as the reference standard because histopathology is the criterion standard for diagnosis of neoplasia. This is an important strength of our systematic review and provides a more realistic and accurate estimate for the index and comparative tests evaluated. In previous studies of accuracy of cytology including both surgical pathology and clinical follow-up[39] as reference standard, pooled sensitivities were 12% higher than in studies with exclusive surgical pathology[40] as reference standard in the diagnosis of mucinous cysts, with overestimation of test accuracy.

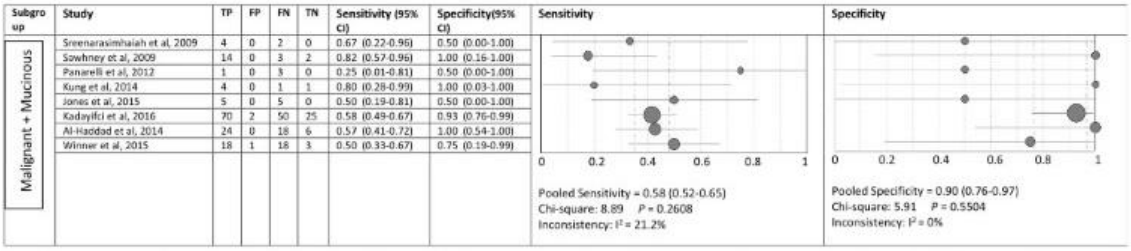


FIGURE 6. Forest plots of the studies included for CEA of greater than 192 ng/mL. In parentheses are the 95% CIs of the sensitivity and specificity. The figure shows the estimated sensitivity of the study (circle) and its 95% CI (horizontal line). The area of the circle reflects the weight that the study contributes to the meta-analysis.

Limitations of this study include incomplete reporting in DTA in primary studies, with no separate information for distinction of malignant and mucinous cysts in 2 studies[5,31] and in another 2 studies for distinction of benign and premalignant mucinous cysts.[29,33] These 4 studies were included in the group of 7 studies with more patients analyzed in the meta-analysis. Another limitation is the time elapsed between the index tests and the reference standard. The final diagnosis could have been made at different time intervals from the tests. If the time between index tests and reference standard is too long, the true diseased status of the patient may have changed by the time the reference standard was assessed. Finally, the low number of malignant cysts per study (0–13), except for 4 studies,[5,7,9,30] may contribute to part of the heterogeneity in the sensitivity observed.

Future Perspectives

With the increasing diagnosis of asymptomatic PCNs, some with malignant potential, there is a growing need to find accurate biomarkers of malignancy in these lesions, to reduce surgeries on benign cysts and still diagnose and resect early malignant lesions with favorable prognosis. DNA molecular markers, particularly *KRAS* mutation, which is an early event in pancreatic carcinogenesis, have the potential to fulfill this need, but clinicians should be aware of their current limitations in diagnostic performance and type of lesions identified.

Certainly, the significant costs, logistic difficulties in collecting and preserving material for future molecular analysis in busy general hospitals, and the technical complexity of the test make its generalized use difficult in clinical practice. Moreover, large multicenter validation studies are still missing.

Additionally, there is a need for more trials to confirm their clinical relevance in patient outcomes, such as early cancer diagnosis, number of surgeries of benign lesions avoided, and prognostic value in numerous cysts that require periodic surveillance.

Moreover, for successful massive implementation of molecular markers in pancreatic cyst clinics, a validation of *KRAS* mutation as a complementary test to patients with an unavailable CEA level and a nondiagnostic cytology will be insufficient. Its development as a universal, highly accurate, first-line test with clinical impact in cyst diagnosis and patient management will be required. Next-generation sequencing reliably allows analysis of multiple gene panels both in PCF and peripheral blood and offers an attractive option to increase the accuracy of molecular analysis in diagnosis and risk stratification of these lesions.[42]

Finally, with current evidence, *KRAS* can only be recommended as a second-line test in the case that CEA and cytology of PCF are nondiagnostic. It would be useful to determine the additional value of the *KRAS* in combination with the other tests and to evaluate the adequate order of the tests, in order to maximize the diagnoses of malignant and/or significant cysts.

CONCLUSIONS

The intended use and clinical role of *KRAS* mutational analysis in the present should be limited to patients with an undefined CEA level and a nondiagnostic cytology, serving only as a complementary diagnostic test due to its limited accuracy. *KRAS* has lower diagnostic accuracy than CEA and cytology and should not replace standard EUS-FNA analysis. Clinicians should be aware of a significant rate of FP results of *KRAS* mutation if the diagnosis of a malignant cyst is under consideration.

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META-ANALYSIS

Genetic testing *vs* microforceps biopsy in pancreatic cysts: Systematic review and meta-analysis

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Abstract

BACKGROUND

Carcinoembryonic antigen (CEA) and cytology in pancreatic cystic fluid are suboptimal for evaluation of pancreatic cystic neoplasms. Genetic testing and microforceps biopsy are promising tools for pre-operative diagnostic improvement but comparative performance of both methods is unknown.

AIM

To compare the accuracy of genetic testing and microforceps biopsy in pancreatic cysts referred for surgery.

METHODS

We performed a literature search in Medline, Scopus, and Web of Science for studies evaluating genetic testing of cystic fluid and microforceps biopsy of

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pancreatic cysts, with endoscopic ultrasound with fine-needle aspiration (EUS-FNA) prior to surgery and surgical pathology as reference standard for diagnosis. We evaluated the diagnostic accuracy for: 1- benign cysts; 2- mucinous low-risk cysts; 3- high-risk cysts, and the diagnostic yield and rate of correctly identified cysts with microforceps biopsy and molecular analysis. We also assessed publication bias, heterogeneity, and study quality.

RESULTS

Eight studies, including 1206 patients, of which 203 (17%) referred for surgery who met the inclusion criteria were analyzed in the systematic review, and seven studies were included in the meta-analysis. Genetic testing and microforceps biopsies were identical for diagnosis of benign cysts. Molecular analysis was superior for diagnosis of both low and high-risk mucinous cysts, with sensitivities of 0.89 (95%CI: 0.79-0.95) and 0.57 (95%CI: 0.42-0.71), specificities of 0.88 (95%CI: 0.75-0.95) and 0.88 (95%CI: 0.80-0.93) and AUC of 0.9555 and 0.92, respectively. The diagnostic yield was higher in microforceps biopsies than in genetic analysis (0.73 *vs* 0.54, respectively) but the rates of correctly identified cysts were identical (0.73 with 95%CI: 0.62-0.82 *vs* 0.71 with 95%CI: 0.49-0.86, respectively).

CONCLUSION

Genetic testing and microforceps biopsies are useful second tests, with identical results in benign pancreatic cysts. Genetic analysis performs better for low- and high-risk cysts but has lower diagnostic yield.

Key words: Pancreatic cysts; Endoscopic ultrasound; Endoscopic ultrasound with fine-needle aspiration; Genetic testing; Microforceps biopsy; Molecular analysis; KRAS; Carcinoembryonic antigen; Cytology

Core tip: With the increasing diagnosis of asymptomatic pre-malignant pancreatic cysts, there is a growing need for accurate and affordable diagnostic tests. The goal is to detect and resect early malignancy, while avoiding unnecessary follow-up in benign cysts and surgery in low-risk cysts. Genetic testing is promising, but with current diagnostic limitations, significant costs, logistic difficulties in preserving material for future analysis, and technical complexity, its generalized use seems difficult. If microforceps biopsy proves in larger studies to be safe and to allow correct diagnosis, it may be immediately implemented, because the endoscopic procedure is standard, and histology is widespread in clinics.

Citation: Faias S, Pereira L, Luís Â, Chaves P, Cravo M. Genetic testing *vs* microforceps biopsy in pancreatic cysts: Systematic review and meta-analysis. *World J Gastroenterol* 2019; 25(26): 3450-3467
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DOI: https://dx.doi.org/10.3748/wjg.v25.i26.3450

INTRODUCTION

Pancreatic cystic neoplasms (PCNs) are on the rise in clinics due to an ageing population and the increase in routine use of high-quality abdominal imaging[1]. PCNs are generally classified into two main groups: mucinous cystic neoplasms (MCNs) and non-mucinous cystic neoplasms (NMCN). MCNs include intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystadenomas, which are precursor lesions of pancreatic carcinoma, and may be low-risk (pre-malignant with low or intermediate-grade atypia) or high-risk: pre-malignant with high-grade atypia (HGA) or malignant, including adenocarcinomas secondarily cystic. NMCNs include serous cystadenomas and inflammatory cysts (pseudocysts), mostly benign cysts, but may include some rare lesions, considered high-risk as cystic neuroendocrine tumors (cNETs), and acinar cell cystadenomas (ACCs). The heterogeneity in malignant

potential, increased frequency, and significant morbidity and mortality of surgical treatment, makes pre-operative diagnosis of PCNs essential for management. The treatment options for PCNs encompass surgery or conservative surveillance for MCNs, according to malignancy risk, or no further evaluation for most NMCNs.

The differentiation between MCNs and NMCNs is critical, because a misdiagnosis of a MCN can lead to a missed opportunity to treat pancreatic cancer in an early stage and a misdiagnosis of NMCN can result in unnecessary surgery or surveillance with associated morbidity, costs, and negative impact on quality of life.

Currently, morphologic characterization of PCNs and pancreatic cystic fluid (PCF) analysis for carcinoembryonic Antigen (CEA) and cytology are central in diagnosis. A CEA level ≥ 192 ng/mL is the most accurate diagnostic test for MCNs and cytology is highly specific for malignancy[2], but with suboptimal results in large studies with surgical pathology as the gold standard[3]. In fact, a significant part of these lesions remains indeterminate and incorrect pre-operative diagnosis occurs in one third of patients[4,5], making new reliable diagnostic tools urgently needed.

In the last decade numerous studies have shown that genetic analysis of aspirates obtained by EUS-FNA provided a better characterization of PCNs than CEA and cytology[6-14]. Next-generation sequencing (NGS) is a very sensitive technique for detection of genetic mutations that allows the rapid detection of mutations in pre-defined panels of cancer genes, even in samples with limited DNA content, such as PCF. NGS requires storage, infrastructure, data processing, and expert personnel. Moreover, to be cost-effective, large numbers of samples need to be processed, making it applicable only in large centralized laboratories. These reasons make the implementation of NGS in clinical practice still a matter of debate.

The clinical need of better diagnostic tests in PCNs has recently led to the development of a through-the-needle miniature biopsy device for use during EUS-FNA[15,16]. The Moray micro forceps biopsy (MFB) device (US Endoscopy, Mentor, Ohio) is disposable and can pass through a standard 19-gauge EUS-FNA needle that is already used routinely. It allows tissue sampling from the cyst wall, septa or mural nodules and the obtention of a histological evaluation of the epithelial architecture and subepithelial stroma[17]. Adding to the high technical success and excellent safety profile[18,19], the new device has shown to improve the diagnostic accuracy of specific cyst subtypes[20,21]. Another major advantage of MFB is the simultaneous tissue sampling and PCF acquisition, with just an additional histologic analysis that follows standard definitions and is already routine in clinics.

The aim of this systematic review and meta-analysis is to evaluate the diagnostic performance of molecular analysis (MA) and MFB and find the most robust additional diagnostic technique in PCNs, in the pre-operative setting.

MATERIALS AND METHODS

This systematic review and meta-analysis is conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis of Diagnostic Test Accuracy Studies, the PRISMA-DTA Statement[22], and the protocol is registered at PROSPERO (CRD42018111910).

Literature search and study selection

A comprehensive search of databases, including Medline, Scopus, and Web of Science, for the past 8 years (January 1st, 2010 to July 31st, 2018) and restricted to human studies was performed. No language restrictions were applied. The following search terms were used in two independent searches: "pancreas", "cyst", "molecular", "analysis"; and "micro", "forceps", "microforceps", "biopsy". A search of related articles was performed, adding additional studies. Duplicate articles, reviews, trials including other kinds of neoplasms, and trials with molecular markers not compliant with the defined inclusion criteria were removed. The references of all selected studies were hand-searched for additional articles.

Inclusion criteria: Published studies were included in the meta-analysis if they analyzed: (1) Patients with symptomatic or incidental pancreatic cysts with a definitive surgical pathology diagnosis; (2) Genetic mutations performed with high sensitive techniques, such as NGS in PCF obtained by EUS-FNA prior to surgery; (3) At least four genetic mutations, including *KRAS, GNAS, VHL*, and at least another genetic mutation representative of aggressive neoplasms (*PIK3CA, TP53, SMAD4, PTEN, CDKN2A*); (4) PCNs evaluated by EUS-FNA with MFB for diagnosis; and (5) Surgical pathology specimens with available data.

Exclusion criteria: (1) Studies of MA with fewer than the four genetic mutations

previously defined; (2) Studies involving solid pancreatic lesions; (3) Studies using PCF not obtained by EUS-FNA; (4) Reviews, case reports, case series with fewer than five patients, letters to editor, exploratory studies, and papers published only in abstract form; (5) Studies with cytology and clinical surveillance as standard of diagnosis. Two authors (SF and AL) independently judged study eligibility and disagreements were resolved by consensus.

Histological criteria: We classified the PCNs of the included studies into three main groups: (1) High-risk cysts (adenocarcinoma or high grade dysplasia in IPMNs and MCNs, secondarily cystic adenocarcinomas, cNETs, and ACCs; (2) Low-risk mucinous cysts (IPMNs and MCNs with intermediate or low-grade dysplasia); and (3) Benign cysts (SCAs, pseudocysts, and other rare cysts (RCs) included in some articles, as retention cysts, lymphoepithelial cysts, epidermoid cysts, squamoid cysts).

Tests under investigation: The index tests were: (1) MA of PCF; and (2) MFB of PCNs, including cyst wall, septs, and nodules. A diagnosis of cNET or ACC does not warrant a malignancy diagnosis, but surgery is recommended in surgically fit patients. Due to a recommendation of identical treatment to malignant and mucinous high-risk cysts, for the purpose of analysis in this study, each one of these diagnoses was classified as a high-risk cyst.

Data extraction

After study selection, two authors (SF and AL) extracted and registered the data from each study onto a standardized worksheet. Disagreements were discussed and reviewed by a third author (LP). The data retrieved were: first author, publication year, study period and design (prospective or retrospective), reference for diagnosis, sample size (all patients included in the study), technical success, adverse events, diagnostic yield, surgical cohort (number of patients with a surgical pathology specimen), cyst size, cyst location, specific cyst types, number of high-risk cysts, mucinous low-risk and benign cysts diagnosed by MA and MFB comparing to surgical pathology specimens. In the MFB studies, technical success was defined as the ability to puncture the cysts and perform the biopsies; and the diagnostic yield was defined as the ratio between the number of patients included in the study and the patients in whom enough material allowed the acquisition of a histopathologic diagnosis. In the MA group, diagnostic yield was defined as a ratio between the number of patients included in the study and the number of patients with DNA available to perform molecular analysis in PCF.

Outcomes

The primary outcomes of this study were the data to obtain the accuracies of MA and MFB for the diagnosis of PCNs, including high-risk cysts, mucinous low-risk cysts, and benign cysts. Secondary outcomes were the diagnostic yield of genetic testing and MFB and the number of cysts correctly identified for each of the tests studied.

Quality analysis

Methodological quality of included primary studies was assessed by two authors (SF and AL) using the modified QUADAS-2 tool[23]. The PRISMA-DTA Statement recommendations were used for reporting this systematic review[22,24].

Statistical analysis and data synthesis

The reference standard was a surgical pathology specimen that allowed the classification of PCNs into three defined groups of diagnosis: high-risk cysts, mucinous low-risk cysts, and benign cysts. This resulted in a two-by-three table with correct and incorrect test results in each of the three referenced groups, for each of the tests analyzed, MA and histology were obtained by MFB.

To calculate tests' accuracy and to reflect on the categories that are useful in clinical practice and that guide management, we constructed two-by-two tables, considering three definitions of "relevant" cysts: (1) High-risk cysts – proven malignant cysts, IPMNs, and MCNs with HGA, cNETs, ACCs; Non-High-risk cysts – all cysts except those proven to be high-risk. (2) Low-risk mucinous cysts – proven mucinous low-risk cysts; High-risk cysts – all except those proven to be mucinous low-risk or benign. And (3) Non-benign cysts – all cysts except those proven to be benign; Benign cysts – proven benign cysts.

The ability of the tests to discriminate "relevant" and "non-relevant" cysts using the three definitions of "relevant cysts" was evaluated and the accuracy of the two tests was compared.

The data of the two-by-two tables were used to calculate sensitivity and specificity for each study. We present individual study results graphically by plotting the estimates of sensitivity and specificity (and their 95% confidence intervals (CI)) in

both forest plots and on the summary receiver operating characteristic (sROC) curve plots. The area under the curve (AUC) is equal to 1 for a perfect test and 0.5 for a completely uninformative test. The AUC is equal to the probability that if a pair of relevant and non-relevant cysts is selected at random, the relevant cyst will have a higher test result than the non-relevant cyst. Pooled estimates of the sensitivity and specificity were obtained by the DerSimonian-Laird method (random effect model) to incorporate variation among studies, when data are heterogeneous. Otherwise, we used the Mantel-Haenszel method (fixed effect model).

Heterogeneity was investigated in the first instance through visual examination of forest plots of sensitivities and specificities and through visual examination of the ROC plot of the raw data. Last, we used statistical tests, including chi-square and Cochran-Q to evaluate if the differences across the studies were greater than expected by chance alone. A low P value suggests presence of heterogeneity. In addition to these statistics we used the statistic I^2 of Higgins, which has been proposed as a measure to quantify the amount of heterogeneity[25,26]. The scale of I^2 has a range of 0 to 100% and values on the order of 25%, 50% and 75% are considered low, moderate, and high heterogeneity, respectively.

Another goal of this work was to obtain, for each of the tests, the correctly identified cyst rate and the diagnostic yield in predicting a histopathologic diagnosis.

We used Comprehensive Meta-Analysis software (Version 2.0) for assessment of diagnostic yield of the tests and Meta-DiSc (version 1.4 – Meta-Analysis of Diagnostic and screening tests[27]) to obtain the accuracy of each of the tests.

RESULTS

Systematic Review

Our search revealed 16 study titles and abstracts for MFB and 264 titles for MA. In Figure 1A and B are described the selection process of the articles included in this study. After all steps, eight studies were considered suitable for qualitative and seven for quantitative analysis. We excluded 20 full-text articles after review, because they were case series of two patients[16] (n = 1), exploratory or pilot studies[28,29] (n = 2), no information of mutation status was available[30] (n = 1), pancreatic cystic fluid was obtained during surgery[31] (n = 1), insufficient or absent data of cysts with surgical pathology diagnoses[12,32,33] (n = 3), and mutations only of *KRAS* and/or *GNAS*[14,34-44] (n = 12).

Of the eight studies that met the inclusion criteria, design was retrospective in six and prospective in two, all were published from 2015 to 2018. These eight studies included a total of 1206 patients, of which 203 (17%) underwent surgical resection and a surgical pathology specimen was available as reference standard and included in the analysis. We excluded all patients with cytology and clinical follow-up data, but for whom a surgical pathology specimen was not available. The characteristics of the studies, surgical pathology diagnoses, and MA and MFB results are presented in Tables 1[32,45-47] and 2[18-21].

Quality assessment and publication bias: Methodological quality of primary studies included was assessed by two authors (SF and AL) using the modified QUADAS-2 tool[23], which evaluates the quality of articles for systematic reviews of diagnostic accuracy studies in four domains, including patient selection, index test, reference standard, and flow and timing, for risk of bias and applicability concerns. Results are presented in Figure 2, which was sketched with templates available at www.quadas.org. The studies included in this review all showed a "low-risk" classification as the index tests (MA and MFB) and the reference standard (surgical pathology specimen) were reliable and mentioned in all studies. However, a "high-risk" of selection bias was demonstrated in patient selection (neither random nor sequential patients included in several studies) and in flow and timing because only a small proportion of the patients evaluated in all studies, except one, were included in the analysis. In fact, most patients were excluded in all studies as the inclusion criteria requiring surgical pathology as diagnostic reference were not met. Applicability concerns in patient selection were also significant in all studies, because the subgroup of PCNs referred for surgery is more often malignant than PCNs on surveillance, which would also be targeted with this review. Because of this bias, there may be an overestimation of both the sensitivity of the index tests, due to a more severe spectrum of PCNs that are referred for surgery, and the positive predictive value (PPV) for diagnosis of high-risk cysts, due to an increased prevalence of malignant cysts in a surgical cohort of PCNs.

Meta-analysis

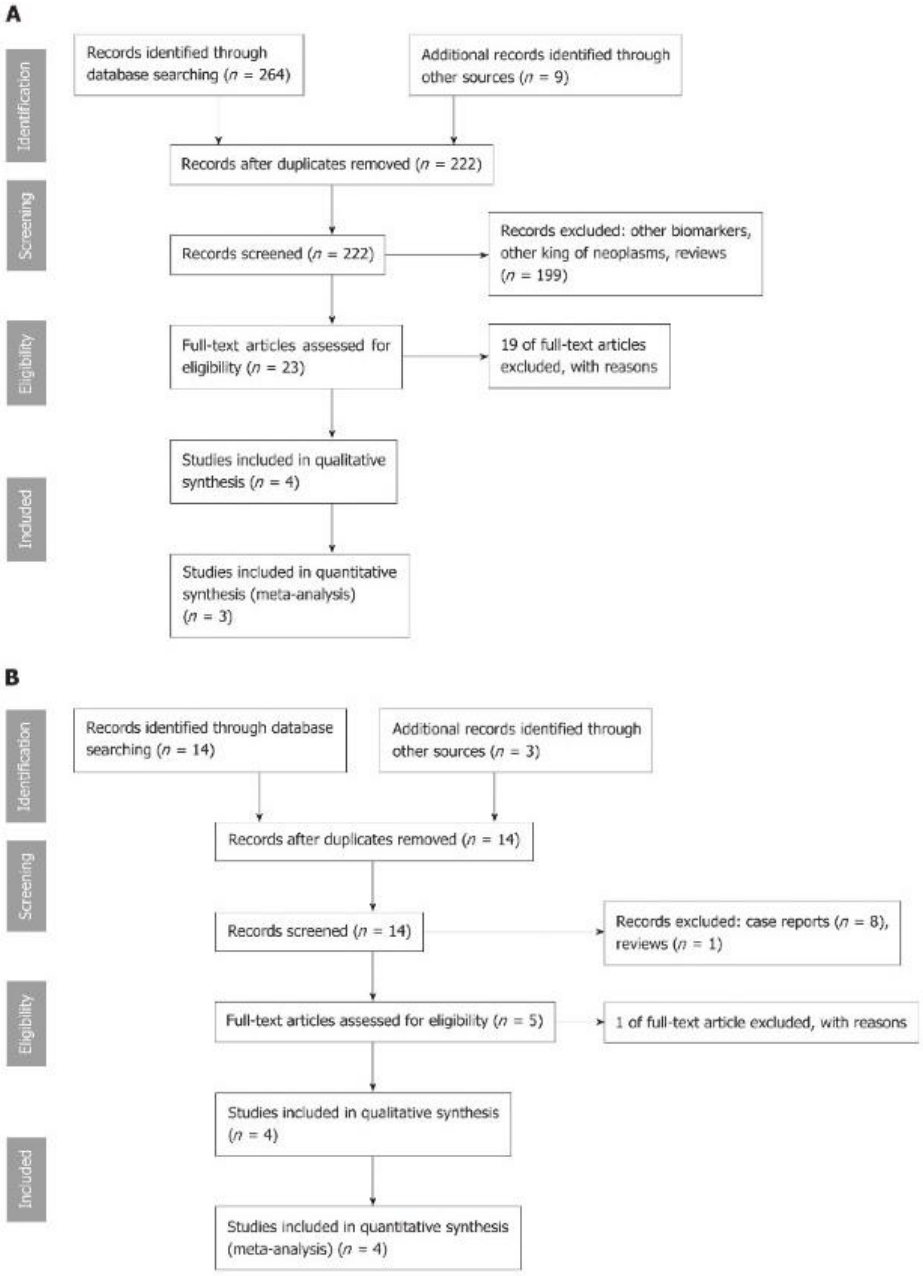


Figure 1 Flowchart with identification of eligible studies. A: Molecular analysis; B: Microforceps biopsy.

Molecular analysis: Four articles were included in the meta-analysis for diagnostic accuracy of MA. For each of the three definitions of relevant cyst, forest plots of sensitivity and specificity with heterogeneous denoted are shown in Figure 3.

The three criteria to define "relevant cysts" resulted in a different range of the specificity and sensitivity of the studies included as shown in Figure 3. For diagnosis of the subgroup with high-risk and low-risk mucinous cysts that require intervention (either surgery or surveillance) comparing to benign cysts the pooled sensitivity was 0.75 (95%CI: 0.66-0.83) and the pooled specificity was 0.72 (95%CI: 0.56-0.85) for MA. In the subgroup of high-risk cysts that require surgery, comparing to other cysts

A

Study	Risk of bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Jones *et al*[45], 2015	☺	☺	☺	☹	☹	☺	☺
Singhi *et al*[46], 2016	☺	☺	☺	☹	☹	☺	☺
Rosenbaum *et al*[22], 2016	☹	☺	☺	☺	☹	☺	☺
Singhi *et al*[47], 2017	☺	☺	☺	☺	☹	☺	☺
Mittal *et al*[13], 2018	☹	☺	☺	☺	☹	☺	☺
Zhang *et al*[10], 2018	☹	☺	☺	☺	☹	☺	☺
Basar *et al*[21], 2018	☹	☺	☺	☺	☹	☺	☺
Kovacevic[19], 2018	☹	☺	☺	☺	☹	☺	☺

☺ Low risk; ☹ High risk; ? Unclear risk.

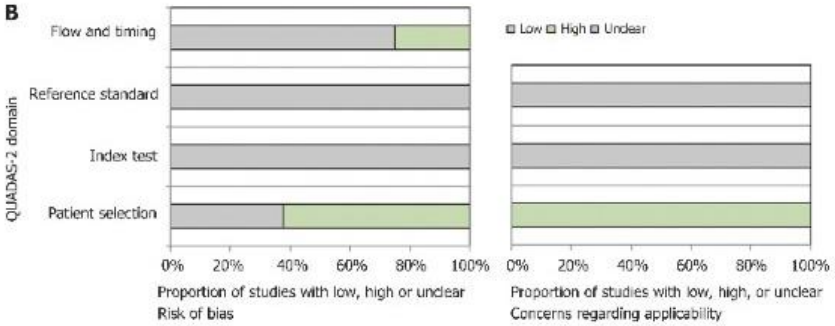


Figure 2 Quality assessment of the studies using QUADAS-2. A: Tabular presentation of risk bias for each study; B: Graphical display of bias.

requiring conservative management, the sensitivity was 0.57 (95%CI: 0.42-0.71) with a specificity of 0.88 (95%CI: 0.80-0.93). In the subgroup of low-risk mucinous cysts comparing to high-risk, the pooled sensitivity was 0.89 (95%CI: 0.79-0.95) and the pooled specificity was 0.88 (95%CI: 0.75-0.95).

Figure 4 displays the sROC curves of MA, showing the sensitivity of the individual articles mapped on the vertical scale, 1-specificity on the horizontal scale, with the summary (sensitivity, 1-specificity) point marked, as well as the summary ROC curve and the confidence region for the summary (sensitivity, 1-specificity) points. The area under the sROC curve was 0.7706 (SE: 0.0927) in non-benign cysts, 0.9248 (SE: 0.0691) in high-risk cysts, and 0.9555 (SE: 0.0293) in mucinous low-risk cysts. The results of the studies had greater variation in non-benign cysts as shown by the wide confidence region.

In the four studies, 566 patients had DNA available to perform MA in PCF. Pooled analysis (Figure 5) showed a diagnostic yield of 54.3% (95%CI: 49.8%-58.7%; I^2 = 39.605%; test for heterogeneity P = 0.174).

By considering the classification of cysts by specific type (IPMNs, MCNs, cNETs, SCAs, pseudocysts, ACCs, and other RCs), MA identified correctly 73.1% of cysts (95%CI: 61.6%-82.2%; I^2 = 37.381%; test for heterogeneity P = 0.203) (Figure 5).

Micro forceps biopsy: Four articles were included in the meta-analysis for diagnostic accuracy of histology obtained using MFB. Figure 6 shows the forest plots of sensitivity and specificity for the three subgroups of relevant cysts. The forest plots for MFB show variable specificities within the papers, from 0 to 1, which can be due to the small numbers of patients with the target condition in some studies.

For each of the three subgroups there exists a low heterogeneity in sensitivity (I^2 = 0%, I^2 = 21.4%, I^2 = 0%) and specificity (I^2 = 0%, I^2 = 0%, I^2 = 21.4%), therefore fixed effect models were used. As presented in Figure 6, in the first subgroup the pooled sensitivity was 0.73 (95%CI: 0.50-0.89) and the pooled specificity was 0.88 (95%CI: 0.28-1.00). In the second subgroup sensitivity was 0.81 (95%CI: 0.46-0.98) with a specificity of 0.77 (95%CI: 0.50-0.94) and in the last subgroup the pooled sensitivity was 0.64 (95%CI: 0.33-0.88) and the pooled specificity was 0.81 (95%CI: 0.46-0.98).

The results were plotted as a symmetrical sROC curve (Figure 4). The area under the sROC curve was 0.7640 (SE: 0.1261) in the first subgroup, 0.8154 (SE: 0.098) in the second subgroup, and 0.7509 (SE: 0.1277) in the last subgroup.

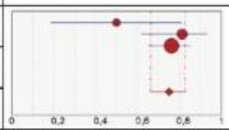
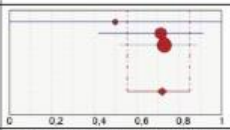
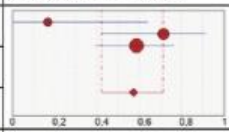
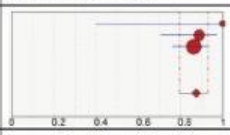
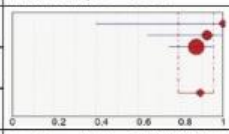
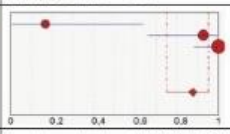
Subgroup	Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity	Specificity
High risk + Low Risk *vs* benign	Jones *et al*[45], 2015	5	0	5	0	0.50 (0.19-0.81)	0.50 (0.00-1.00)		
	Singhi *et al*[36], 2016	22	4	5	10	0.81 (0.62-0.94)	0.71 (0.42-0.92)		
	Singhi *et al*[47], 2017	58	7	18	19	0.76 (0.65-0.85)	0.73 (0.52-0.88)		
								Pooled sensitivity = 0.75 (0.66-0.83)	Pooled specificity = 0.72 (0.56-0.85)
								Chi-square: 3.59; P = 0.1661	Chi-square: 0.23; P = 0.8903
								Inconsistency: I^2 = 44.3%	Inconsistency: I^2 = 0%
High Risk *vs* Non High Risk	Jones *et al*[45], 2015	1	0	5	4	0.17 (0.00-0.64)	1.00 (0.40-1.00)		
	Singhi *et al*[36], 2016	10	3	4	24	0.71 (0.42-0.92)	0.89 (0.71-0.98)		
	Singhi *et al*[47], 2017	17	10	12	63	0.59 (0.39-0.76)	0.86 (0.76-0.93)		
								Pooled sensitivity = 0.57 (0.42-0.71)	Pooled specificity= 0.88 (0.80-0.93)
								Chi-square: 5.43 P-value = 0.0662	Chi-square: 1.21 P-value = 0.5458
								Inconsistency: I^2 = 63.2%	Inconsistency: I^2 = 0%
Low Risk *vs* High Risk	Jones *et al*[45], 2015	4	5	0	1	1.00 (0.40-1.00)	0.17 (0.00-0.64)		
	Singhi *et al*[36], 2016	12	1	1	13	0.92 (0.64-1.00)	0.93 (0.66-1.00)		
	Singhi *et al*[47], 2017	41	0	6	29	0.87 (0.74-0.95)	1.00 (0.88-1.00)		
								Pooled sensitivity = 0.89 (0.79-0.95)	Pooled specificity = 0.88 (0.75-0.95)
								Chi-square: 1.24; P = 0.5391	Chi-square: 23.82; P < 0.0001
								Inconsistency: I^2 = 0%	Inconsistency: I^2 = 91.6%

Figure 3 Forest plots of the studies included for molecular analysis. In parentheses are the 95% confidence intervals (CI) of the sensitivity and specificity. The figure shows the estimated sensitivity and specificity of the study (red circle) and its 95% CI (blue horizontal line). The area of the circle reflects the weight that the study contributes to the meta-analysis.

By pooling the data of the four studies that investigated the use of MFB to obtain a histopathologic diagnosis, we obtained a diagnostic yield of 73.1% (95%CI: 61.4%-82.2%; I^2 = 47.774%; test for heterogeneity P = 0.125) (Figure 5).

By considering the outcome "specific cyst type" diagnosis, MFB correctly identified 70.7% of the cysts (95%CI: 49.4%-85.6%; I^2 = 0%; test for heterogeneity P = 0.056) (Figure 5).

DISCUSSION

In this meta-analysis we analyzed two different but promising tests to diagnose PCNs – molecular analysis and microforceps biopsy. To our knowledge this is the first study of this nature, and it included 1206 patients with PCNs of which 1058 underwent MA and 148 MFB. All patients had the index tests performed in PCF obtained pre-operatively, exclusively with NGS for MA and the Moray micro forceps biopsy device (US Endoscopy, Mentor, Ohio) used for MFB. We analyzed 203 cysts, 178 evaluated with MA and 25 with MFB, all referred for surgery, and with a surgical pathology specimen used as reference standard for diagnosis.

In this comparative analysis we included all studies, without restriction to simultaneous evaluation of both tests, because only one of such studies has been published[29]. This study, which includes 48 patients but only 10 surgical pathology specimens, showed identical results for MA and MFB in low-risk and high-risk cyst diagnosis, but higher specific cyst type diagnosis for MFB.

The data from the seven studies included in the meta-analysis, although with limited number of patients, particularly for MFB, suggests that MA is more accurate than MFB for diagnosis of PCNs, including high-risk and low-risk lesions. MA has superior accuracy to discriminate high-risk cysts from other PCNs and low-risk from high-risk neoplastic cysts. MA performance was considered excellent with AUC values of 0.92 and of 0.96 for high-risk and low-risk neoplastic lesions, respectively, as compared to MFB, which showed a fair or good performance, with an AUC of 0.81 and 0.75, respectively for the same lesions (Figure 4). The specificity of MA is good (0.88) but it has a low sensitivity (only 0.57) for high-risk cysts. This may be explained

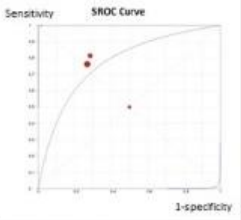
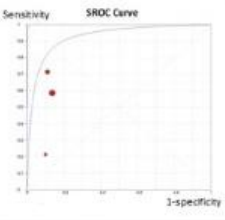
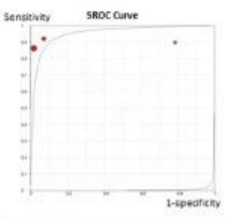
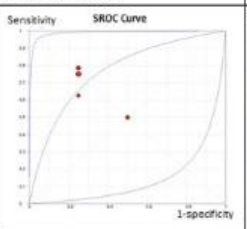
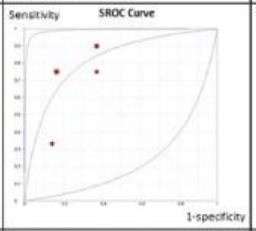
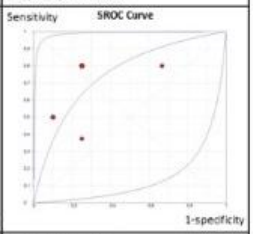
Test	sROC curves		
	(High risk + Low risk) *vs* benign	**High risk *vs* (benign+low risk)**	**Low risk *vs* High risk**
Molecular analysis			
	Symmetric sROC	Symmetric sROC	Symmetric sROC
	AUC = 0.7706	AUC = 0.9248	AUC = 0.9555
	SE (AUC) = 0.0927	SE (AUC) = 0.0691	SE (AUC) = 0.0293
MicroForceps biopsies			
	Symmetric sROC	Symmetric sROC	Symmetric sROC
	AUC = 0.7640	AUC = 0.8154	AUC = 0.7509
	SE (AUC) = 0.1261	SE (AUC) = 0.098	SE (AUC) = 0.1277

Figure 4 Summary receiver operating characteristics plots. ROC: Receiver operating characteristic curve; AUC: Area under the curve; SE: Standard error.

by technical issues, by low prevalence of relevant genetic mutations in malignant PCNs, or by mutations not included in the current NGS panels. The sensitivity and specificity are high (0.89 and 0.88, respectively) for MA when comparing low-risk to high-risk cysts, which reflects the genetic nature of pancreatic carcinogenesis with cumulative mutations from benign to malignant cysts[48].

For discriminating benign cysts from both low-risk and high-risk cysts, the performance of MA and MFB was identical and fair according to AUC values of 0.77 and 0.76, respectively. This non-superiority of MA in the diagnosis of benign cysts in this meta-analysis may be due to technique-inherent issues and/or under-representation of benign cysts in surgical series. In fact, "no genetic mutation" is considered a false negative result in most benign rare cysts, but some of these lesions (retention cysts, *etc*.) have no diagnostic genetic mutations. On the contrary, the most frequent benign cysts, SCAs, harbor a VHL mutation, exclusively present in these benign lesions and allowing for discarding a malignant lesion. In the MA studies, one third of rare benign cysts were classified as false negative results, due to absence of characteristic mutations (Table 1). Another example of PCN that is not amenable to a MA diagnosis with current genetic panels is cNET, also reducing the accuracy of MA for diagnosis of high-risk cysts. The sensitivities were identical for MA and MFB (0.75 and 0.72), but the latter had higher specificity (0.73 and 0.88, respectively). Limited tissue sampling with MFB can explain the reduced sensitivity with robust specificity. As MA depends on denuded DNA in suspension in PCF, no sampling error is expected, which may explain its greater accuracy in neoplastic cysts, comparing to MFB.

Concerning secondary outcomes, even with the limitations of tissue sampling inherent to MFB, this meta-analysis showed that the diagnostic yield of MFB was superior to MA with rates of correctly identified cyst identical with MA and MFB (Tables 1 and 2). In fact, the definition of diagnostic yield, which for MA was "detection of genetic mutations", may have led to a falsely low value due to the presence of some rare types of benign cysts (retention cysts, lymphoepithelial cysts, epidermoid cysts, squamous cysts in two studies[46,47]) that have no characteristic diagnostic genetic mutations.

In clinical practice, patient symptoms, cyst imaging features, CEA, and cytology of PCF are required for diagnosis and decision for either treatment or surveillance according to cyst types[49]. PCF analysis, including CEA to distinguish mucinous from

Table 1 Characteristics of studies of molecular analysis included in the analysis

Ref.	Type of study (study period)	Number of genes/ DNA quantity, ng/microL (mean and interval)	Diagnosis	Sample size (all patients in the study)	Technical success (successful molecular analysis)	Adverse events (major/ minor)	Diagnostic yield (patients with DNA mutations detected)	Surgical cohort	Mean cyst size and range (mm)	Cysts < 3 cm	Cyst location (H/B/T) Cysts with mutations	Specific Cyst Diagnosis (MA/all patients)	Specific Cyst Diagnosis (MA/ surgical pathology)	High risk cysts (MA/ surgical pathology)	Mucinous low-risk cysts (MA/ surgical pathology)	Benign cysts (MA/ surgical pathology)	Specific cyst type (MA/surgical pathology)							
																	IPMN-ADC	IPMN-HG	IPMN-LG	MCN-HG	MCN-LG	SCA	Pseudocyst	NET	Other[a]
Jones *et al*[40], 2015	Prospective (Mar 2013-Feb 2014) Retrospective (Jan 2014-May 2015)	39/12.3 (0-1283)	Pathology or cytology	86	79 (only 3 due to NGS failure)	NA	46	10	NA	NA	NA	72/92	5/10	1/6	4/4	0/0	1/4	0/2	4/4	0/0	0/0	0/0	0/0	0/0
Singhi *et al*[41], 2016		6/NA	Pathology or clinicopathologic	225	225	NA	118	41	2.8 (8-115)	152	95/81/49	130/225	32/41	10/14	12/13	10/14	9/9[b]	1/2[c]	12/12	0/0	0/1	2/2	8/8	0/2	0/5[d]
Rosenbaum *et al*[42], 2016	Retrospective (Mar 2013-Jun 2015)	9/NA	Pathology or clinicopathologic	105	105	NA	67	25	NA	NA	NA	67/113[e]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Singhi *et al*[43], 2017	Prospective (Jan 2014-Ju 2017)	11/6.93 (0.01-248)	Pathology or cytology	642	595	NA	335	102	2.7 (8-210)	NA	320/B+T=306	357/626	77/102	17/29	41/47	19/26	13/13	4/4[b]	39/39	0/2	2/8	2/3	17/17	0/9	0/7[f]
				1058	1004	NA	566 (54%)	178	2.7 (8-210)	152		828/965 (86%)	114/153 (74%)	28/49 (62%)	57/64 (89%)	29/40 (73%)	23/26	5/8	55/55	0/2	2/9	4/5	25/25	0/11	0/12

[a]113 samples of 105 patients; [b]*TP53*, *PIK3CA*, *PTEN*; [c]1 ACC; [d]Includes: ACC, retention cyst, lymphoepithelial cyst, epidermoid cyst, and squamoid cyst. MA: Molecular analysis; NA: Non-available data; ACC: Acinar cell cyst; H/B/T: Head body and tail of the pancreas; ADC: Adenocarcinoma; HG: High-grade; LG: Low-grade; NET: Neuroendocrine tumor.

Table 2 Characteristics of studies of microforceps biopsies included in the analysis

Ref.	Type of study (study period)	Diagnosis	Sample size (all patients in the study)	Technical success (successful cyst puncture and biopsy)	Adverse events (major/minor)	Diagnostic yield (histopathologic diagnosis available)	Surgical cohort	Mean cyst size and range, (mm)	Cysts < 3 cm	Cyst location (H/B/T)	Specific cyst diagnosis (MFB/all patients)	Specific cyst (MFB/surgical pathology)	High risk cysts (MFB/surgical pathology)	Mucinous low risk cysts (MFB/surgical pathology)	Benign cysts (MFB/surgical pathology)	Specific cyst type (MFB/surgical pathology)							
																IPMN-ADC	IPMN-HG	IPMN-LG	MCN-HG	MCN-LG	SCA	Pseudocyst	NET	Other (ACC)
Mittal *et al*[11], 2018	Retrospective (Dec 2016-June 2017)	Pathology, cytology and cyst fluid analysis	27	27	0/0	24	4	38 (15-70)	20	14/B+T=13	24/27	2/4	1/1	1/3	0/0	0/0	0/0	1[1]/3	-	NA	-	-	1/1	-
Zhang *et al*[10], 2018	Retrospective (Jan 2016-Set 2017)	Pathology, cytology and cyst fluid analysis	48	46	0/0	36	10	31 (12-60)	23	13/22/13	24/48	7/10	3/4	4/5	1/1	1[1]/3	1[1]/0	4/4	-	01/1	1/1	-	-	1/1
Basar *et al*[11], 2018	Retrospective (2015-2016)	Pathology, cytology and cyst fluid analysis	42	38	0/2	26	7	28 (12-60)	24	16/17/9	15/42	6/7	4/4	1/2	1/1	2/2	-	1/1	-	0[1]/1	1/1	-	1/1	1/1
Kovacevic *et al*[11], 2018	Retrospective (Sep 2016-Oct 2017)	Pathology or multidisciplinary conference	31	27	1/2	22	4	34 (12-130)	14	NA/NA/NA	24[1]/31	3/4	0/1	2/2	1/1	0/1	-	3[2]/2	-	-	-	1/1	-	-
			148	138	1/4	108/148(73%)	25	33 (12-130)	81		63/148 (42%)	18/25 (72%)	8/10 (80%)	8/12 (67%)	3/3 (100%)	3/6	1/0	8/10	-	0/2	2/2	1/1	2/2	2/2

[1]1 nondiagnostic on MFB; [2]2 nondiagnostic on MFB; [3]1 false negative IPMN associated invasive carcinoma; [4]2 cases with a suspicion of pseudocyst. MFB: Microforceps biopsy; NA: Non-available data; ND: Nondiagnostic; ACC: Acinar cystadenoma; H/B/T: Head body and tail of the pancreas; ADC: Adenocarcinoma; HG: High-grade; LG: Low-grade; NET: Neuroendocrine tumor.

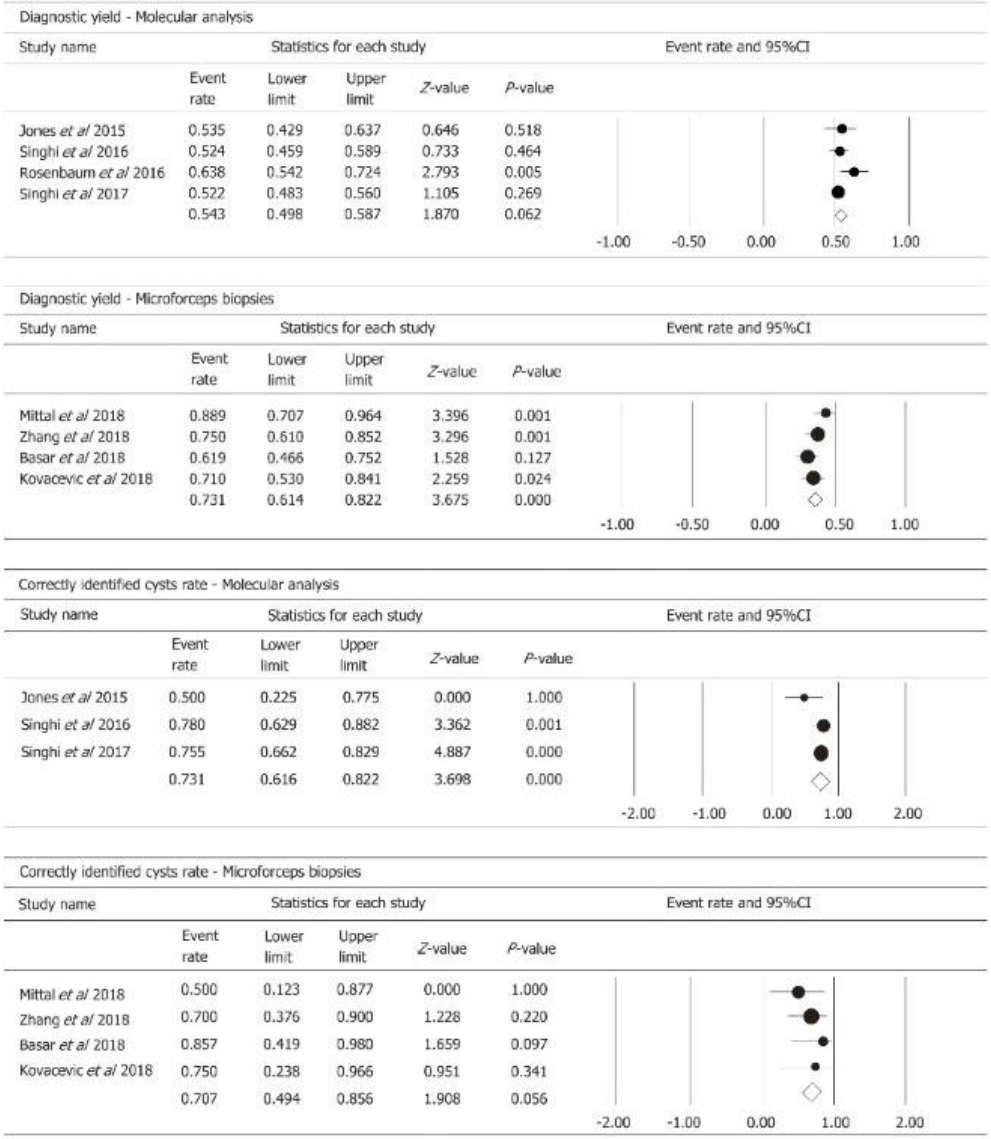


Figure 5 Forest plots of molecular analysis and microforceps biopsies on the secondary outcomes of this meta-analysis.

non-mucinous cysts and cytology to select those that harbor HGA or early pancreatic carcinoma and require surgical treatment, have suboptimal accuracies[3], due to scant cellularity and limited PCF volume. In this context, additional diagnostic tests are necessary to improve cyst classification and refine clinical decision. DNA markers require limited amounts of PCF, increasing the diagnostic yield[32,45,50,51], but with considerable technical complexity and costs. In fact, in routine clinical practice a major pitfall for PCNs diagnosis is the limited volume of PCF obtained, precluding routine pre-operative testing. As DNA analysis requires less volume of PCF, it may become an alternative test in these circumstances. This major advantage of molecular analysis was not possible to evaluate in this meta-analysis, because the volume of cystic fluid obtained in pancreatic cysts was not available in most studies analyzed.

As MA continues to evolve, questions remain about its accuracy, how it influences patient management, and in what order the analysis should be performed to better support clinical decisions. Previous studies[49] have shown that DNA testing combined with clinical features increased PCNs diagnosis compared to either alone. With

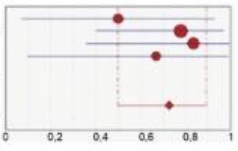
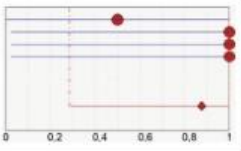
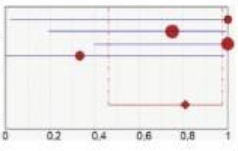
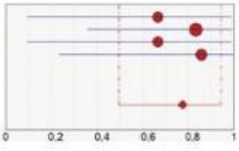
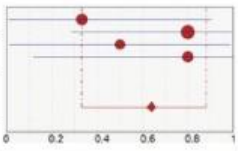
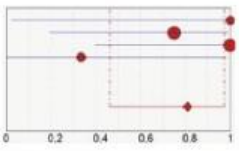
Subgroup	Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity	Specificity
High risk + Low risk *vs* Benign	Mittal *et al*[18], 2018	2	0	2	0	0.50 (0.07-0.93)	0.50 (0.00-1.00)		
	Zhang *et al*[20], 2018	7	0	2	1	0.78 (0.40-0.97)	1.00 (0.03-1.00)		
	Basar *et al*[21], 2018	5	0	1	1	0.83 (0.36-1.00)	1.00 (0.03-1.00)		
	Kovacevic[19], 2018	2	0	1	1	0.67 (0.09-0.99)	1.00 (0.03-1.00)		
								Pooled sensitivity = 0.73 (0.50-0.89)	Pooled specificity = 0.88 (0.28-1.00)
								Chi-square: 1.48 *P*-value = 0.6878	Chi-square: 1.63 *P*-value = 0.6539
								Inconsistency: I^2 = 0%	Inconsistency: I^2 = 0%
High risk *vs* Non high risk	Mittal *et al*[18], 2018	1	1	0	2	1.00 (0.03-1.00)	0.67 (0.09-0.99)		
	Zhang *et al*[20], 2018	3	1	1	5	0.75 (0.19-0.99)	0.83 (0.36-1.00)		
	Basar *et al*[21], 2018	4	1	0	2	1.00 (0.40-1.00)	0.67 (0.09-0.99)		
	Kovacevic[19], 2018	0	0	1	3	0.33 (0.00-0.98)	0.86 (0.23-1.00)		
								Pooled sensitivity = 0.81 (0.46-0.98)	Pooled specificity = 0.77 (0.50-0.94)
								Chi-square: 3.82; *P* = 0.2819	Chi-square: 0.64; *P* = 0.8865
								Inconsistency: I^2 = 21.4%	Inconsistency: I^2 = 0%
Low risk *vs* High risk	Mittal *et al*[18], 2018	1	0	2	1	0.33 (0.01-0.91)	1.00 (0.03-1.00)		
	Zhang *et al*[20], 2018	4	1	1	3	0.80 (0.28-0.99)	0.75 (0.19-0.99)		
	Basar *et al*[21], 2018	1	0	1	4	0.50 (0.01-0.99)	1.00 (0.40-1.00)		
	Kovacevic[19], 2018	2	1	0	0	0.80 (0.12-1.00)	0.33 (0.00-0.98)		
								Pooled sensitivity = 0.64 (0.33-0.88)	Pooled specificity = 0.81 (0.46-0.98)
								Chi-square: 2.24 *P*-value = 0.5246	Chi-square: 3.82 *P*-value = 0.2819
								Inconsistency: I^2 = 0%	Inconsistency: I^2 = 21.4%

Figure 6 Forest plots of the included studies for microforceps biopsies. In parentheses are the 95% confidence intervals (CI) of the sensitivity and specificity. The figure shows the estimated sensitivity and specificity of the study (red circle) and its 95%CI (blue horizontal line). The area of the circle reflects the weight that the study contributes to the meta-analysis.

multiple recent advances in biomarkers, molecular genetics will probably prove to be useful in the management of PCNs[52]. In a previous meta-analysis, pre-operative cytology of PCNs has shown low sensitivity for diagnosis[53], endorsing additional tests to improve diagnosis. Another meta-analysis of diagnostic accuracy of EUS-FNA with CEA and cytology analysis in differentiating mucinous cysts has demonstrated to be accurate to confirm the diagnosis but performed poorly in excluding it[54]. The role of *KRAS* as individual screening test has been analyzed before[55] with poor accuracy and added benefit coming from a combined approach with cytology. A recently published meta-analysis supporting *KRAS, GNAS*, and *RNF43* mutations as diagnostic markers of IPMNs[56] used different methods for mutation detection, different tumor materials, and clinicopathologic data as reference standard for diagnosis, which may limit its clinical application in evaluation of PCNs with mutational analysis performed only in PCF.

In this scenario, new markers are needed for PCNs stratification, and in our meta-analysis both MA and MFB have acceptable diagnostic accuracies. The two largest studies of MA[46,47] showed higher accuracy for diagnosis, which underscores the role of technical aspects of PCF collection, storage, and laboratory analysis for improved accuracy with this technique.

On the other hand, MFB provides tissue fragments for routine histological evaluation, without additional PCF required other than for standard analysis. The technical feasibility of through-the-needle microforceps biopsies revealed to be excellent, even in cysts located in the pancreatic head, despite the required 19-gauge caliber of the EUS-FNA needle. Another potential advantage of MFB is to allow the diagnosis of histologic subtypes of IPMNs, which can potentially be used for risk stratification[57], but still requires further validation.

Strengths and limitations

We applied strict exclusion criteria, with all analyzed patients having a surgical pathology specimen as the reference standard for diagnosis, because histopathology is the gold standard for diagnosis of neoplasia. Another major strength of this meta-analysis is having identical lesions (size and location) analyzed in both groups. These important strengths provide a more realistic accuracy estimate of the tests evaluated. In previous studies of cytology including both surgical pathology and clinical follow-up[34] as reference standard, pooled sensitivities were 12% higher than in studies with exclusive surgical pathology[35] as reference standard in the diagnosis of mucinous cysts, with test accuracy overestimation. Finally, the pooled results have low heterogeneity.

The quality of a systematic review depends on the quality of studies included, and our quality assessment of patient selection regarding the risk of bias and applicability was high. As sensitivity and specificity are sensitive to study design and influenced by the spectrum of disease, sample collection, and processing, there may be a risk of bias and the results, although correct, their interpretation may be inaccurate. Moreover, there was incomplete reporting in one primary study, having no separate information on specific cyst type, mucinous or malignant cyst diagnosis[32], and the study was excluded from quantitative analysis. Although one study was excluded from the meta-analysis, MA with three studies included more patients (953, of whom only 153 in the surgical cohort) than the group of MFB with four studies but fewer patients (148, with only 25 in the surgical cohort). This can represent a surgical selection bias for both tests studied. Moreover, MFB studies were all retrospective, with small sample size, without pathology diagnosis for most benign and pre-malignant cysts, and non-consecutive patients that were selected on endoscopist discretion, which may have led to bias. Another limitation is the time between the index tests and the reference standard, because the final diagnosis could have been made at different time intervals from the tests. If the time between index tests and reference standard is too long, the true disease status of the patient may have changed by the time the reference standard was assessed. Aditionally, the different number of malignant cysts per study, particularly in the MA group, may have led to part of the heterogeneity in sensitivity and specificity. Finally, as MA does not increase the risks of standard EUS-FNA (the analysis is performed in remnant cystic fluid after standard diagnosis) we did not perform a safety analysis of MFB, but the four studies analyzed described only rare non-severe adverse events.

Future perspectives

With the increasing diagnosis of asymptomatic PCNs, most with potential for malignancy, there is a growing need to find accurate and affordable tests for diagnosis. The goal of management of patients with pancreatic cysts is to detect and resect cysts before progression of malignancy, while avoiding unnecessary follow-up procedures in benign cysts and surgery in low-risk PCNs.

Biomarkers of malignancy are promising, but clinicians should be aware of their current diagnostic performance limitations and type of lesions identified. In addition to significant costs, logistic difficulties in preserving material for future molecular analysis in busy general hospitals, and the technical complexity of the test, the generalized use of MA seems difficult in clinical practice. On the other hand, if MFB proves in larger studies to be safe and to allow tissue acquisition and gives the histological criteria needed for a correct diagnosis of PCNs, it may be immediately implemented in clinics, because the endoscopic procedure is standard, and histology is already a widespread procedure in clinics. MFB may be especially useful for benign lesions, for which both surgery and surveillance are unnecessary, representing a considerable burden in pancreas clinics due to current diagnostic limitations[36].

For MA to become relevant in routine clinical care in the future, its role in early cancer diagnosis and its prognostic value in PCNs requiring periodic surveillance must be confirmed. Also, for successful massive implementation, it is required to develop as an universal, highly accurate, first line test with clinical impact in cyst diagnosis, prognosis, and patient management. MA, both in PCF and peripheral blood, for standard analysis of multiple simultaneous biomarkers, allowing non-invasive diagnosis and risk stratification of these lesions[39] would be valuable. For the present time, MA and MFB can only be recommended as complementary or as second line tests in case CEA and cytology of PCF are non-diagnostic. For both tests, large multicenter validation studies are still missing.

CONCLUSION

Our study confirms the diagnostic value of both MA and MFB, with higher diagnostic

accuracy of MA than MFB for both low-risk and high-risk mucinous cysts. Genetic analysis should not be replaced by MFB in this context. Clinicians should be aware of the higher accuracy of MA for the diagnosis of malignant and high-risk cysts.

ARTICLE HIGHLIGHTS

Research background

Carcinoembryonic antigen (CEA) and cytology of pancreatic cystic fluid (PCF) obtained pre-operatively with endoscopic ultrasound with fine-needle aspiration (EUS-FNA) are suboptimal for diagnostic evaluation of pancreatic cystic neoplasms. Genetic testing of PCF and microforceps biopsy obtained by EUS-FNA are promising tools for pre-operative diagnostic improvement. The comparative performance of both methods has not been previously studied.

Research motivation

In the last decade numerous studies have shown that genetic analysis of aspirates obtained by EUS-FNA provided a better characterization of pancreatic cysts than standard CEA and cytology. Next-generation sequencing (NGS) is a very sensitive technique for detection of genetic mutations in pre-defined panels of cancer genes, even in samples with limited DNA content, such as PCF. NGS requires storage, infrastructure, data processing, expert personnel, and large numbers of samples need to be cost-effective. These reasons make the implementation of NGS in clinical practice still a matter of debate. The clinical need of better diagnostic tests in pancreatic cysts led to the development of a through-the-needle miniature biopsy device for use during EUS-FNA. The Moray micro forceps biopsy device (US Endoscopy, Mentor, Ohio) is disposable and can pass through a standard 19-gauge EUS-FNA needle that is already used routinely. It allows tissue sampling from the cyst wall, septa or mural nodules and the obtention of a histological evaluation of the epithelial architecture and subepithelial stroma, with improved pancreatic cyst diagnosis.

Research objectives

To compare the diagnostic accuracy of genetic testing and microforceps in the diagnosis of pancreatic cystic neoplasms referred for surgery.

Research methods

We performed a literature search in Medline, Scopus, and Web of Science for studies evaluating genetic testing of cystic fluid and microforceps biopsy of pancreatic cysts, with EUS-FNA prior to surgery. We used surgical pathology as reference standard for diagnosis. We evaluated the diagnostic accuracy for: benign cysts; mucinous low-risk cysts; high-risk cysts; the diagnostic yield; and rate of correctly identified cysts with microforceps biopsy and molecular analysis.

Research results

Eight studies, including 1206 patients, of which 203 (17%) referred for surgery who met the inclusion criteria were analyzed in the systematic review, and seven studies were included in the meta-analysis. Genetic testing and microforceps biopsies were identical for diagnosis of benign cysts. Molecular analysis was superior for diagnosis of both low and high-risk mucinous cysts. The diagnostic yield was higher in microforceps biopsies than in genetic analysis, but the rates of correctly identified cyst types were identical.

Research conclusions

This study underlines the diagnostic value of both MA and MFB, with higher diagnostic accuracy of MA than MFB for both low-risk and high-risk mucinous cysts. Genetic analysis should not be replaced by MFB in this context. However, MA has higher accuracy in the diagnosis of malignant and high-risk cysts.

Research perspectives

For the present time, MA and MFB can only be recommended as complementary or as second line tests in case CEA and cytology of PCF are non-diagnostic. In the future, for MA to become relevant in routine clinical care, its role must be confirmed, in order to become a first line test with clinical impact in cyst diagnosis, prognosis, and patient management. MA, both in PCF and peripheral blood, for multiple simultaneous biomarkers and non-invasive diagnosis and risk stratification would be valuable. If MFB proves in larger studies to be safe and to allow a correct diagnosis of pancreatic cysts, it may be immediately implemented in clinics. MFB may be especially useful for benign lesions, for which both surgery and surveillance are unnecessary, with uncertain diagnosis due to current diagnostic limitations. For both tests, larger validation studies are missing.

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ORIGINAL ARTICLE

Clinical Impact of *KRAS* and *GNAS* Analysis Added to CEA and Cytology in Pancreatic Cystic Fluid Obtained by EUS-FNA

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Abstract

Background Pancreatic cysts are common incidental findings with malignant potential, raising diagnostic and treatment dilemmas.

Aims To determine the added value of *KRAS* and *GNAS* mutation analysis on cyst classification and decision making.

Methods We analyzed 52 frozen samples of pancreatic cystic fluid obtained by EUS-FNA between 2008 and 2014. In addition to cytology and CEA, mutations of *GNAS* (exons 8 and 9) and *KRAS* (exons 2 and 3) genes were analyzed using Sanger sequencing.

Results There were 52 patients, 67% females, with a mean age of 59 ± 15 years (29–91). Cysts were classified as mucinous in 21 patients (40%) (14 low-risk, seven malignant) and non-mucinous in 31 patients (60%). After EUS-FNA, 11 patients had surgery, six had chemotherapy or palliation, one had endoscopic drainage, and 34 are on follow-up after a mean of 57 months. *KRAS* mutation was detected in nine and *GNAS* in two samples. Patients harboring cysts with *KRAS* mutations were older ($p = 0.01$), cysts were more commonly mucinous ($p = 0.001$) and malignant ($p = 0.01$). *KRAS* mutations were present in both low-risk and malignant mucinous lesions. For identifying mucinous lesions, CEA > 192 ng/mL performed better (AUC ROC = 93%), whereas for malignant/high-risk mucinous lesions, EUS imaging had the best accuracy (AUC ROC = 88%). After molecular analysis, a modification in cyst classification occurred in ten patients, but was correct in only two, a pseudocyst re-classified as IPMN and a malignant cyst as a non-mucinous cyst.

Conclusions In this cohort of patients with pancreatic cysts, *KRAS* and GNAS mutations had no significant diagnostic benefit in comparison with conventional testing.

Keywords EUS-FNA · Pancreatic cystic neoplasm · Genetic analysis · CEA · DNA · KRAS or GNAS

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Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the USA, and it is expected to be the second by 2030. Mucinous cysts are premalignant and would represent an excellent opportunity for early diagnosis in this malignancy [1]. However, the prevalence of pancreatic cysts over 1 cm in the general population is around 2% and cyst prevalence increases with age [2], making differential diagnosis of these lesions a true challenge.

Pancreatic cysts include a wide range of diagnosis and can be non-neoplastic (pseudocysts) or neoplastic, which are the most frequent in clinical practice. According to WHO classification of pancreatic tumors, neoplastic cysts are classified as benign (serous cystadenomas—SCAs), premalignant (intrapapillary mucinous neoplasms—IPMNs and mucinous cystic neoplasms—MCNs, with low-grade epithelial atypia—LG, or high-grade epithelial atypia—HG), and malignant lesions (ductal adenocarcinomas—ADCs, MCNs, and IPMNs with an associated invasive carcinoma—IC, cystic neuroendocrine tumors—NETs, etc.).

In clinical practice, pancreatic cyst management starts with a distinction between non-mucinous from mucinous followed by low-risk versus high-risk/malignant mucinous lesions definition. The Fukuoka guidelines [13] are the gold standard in clinical practice in defining MD-IPMN with a MPD dilation of > 5 mm and "high-risk stigmata" and "worrisome features" that stratify the risk of malignancy in BD-IPMN to recommend resection or increased surveillance. The neoplastic benign/inflammatory cysts (SCAs, pseudocysts) do not progress to malignancy and require, at the most, conservative follow-up. Low-risk mucinous cysts (IPMNs and MCNS with LG) require surveillance due to malignancy risk. In high-risk mucinous/malignant lesions, including premalignant cysts (IPMNs and MCNs—HG) and malignant cysts (ADCs, IPMNs, and MCNs—IC), surgery is indicated. We should be aware that rarely, non-mucinous cysts can be malignant (NETs) and require surgery. Cross-sectional imaging is not accurate in distinguishing different cyst types [3], and cystic fluid analysis with CEA determination and cytology is recommended despite limitations on pancreatic cyst discrimination [4–7].

Molecular analysis in pancreatic cystic fluid (PCF), particularly *KRAS* and *GNAS* mutational analysis, showed promising results in mucinous cysts diagnosis [8]. Some studies show discrepant results [9, 10], and the molecular markers impact in clinical practice remains unclear [11].

The aim of this retrospective cohort study was to evaluate *KRAS* and *GNAS* mutational status in pancreatic cysts in a cohort of prospectively followed patients, to determine its accuracy in identifying mucinous cysts, particularly high-risk/malignant lesions, and to evaluate its added value in decision-making.

Methods

This retrospective study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1143). Cases were selected from the registry of EUS-FNA and PCF database of the Portuguese Institute of Oncology in Lisbon started in 2008, which is used for diagnosis and clinical management of patients with pancreatic cysts in our institution. All patients gave informed consent for EUS-FNA and PCF analysis and storage. After undergoing EUS-FNA, patients are evaluated in pancreas clinic and referred for surgery (surgical cohort) or imaging follow-up, palliation, or endoscopic drainage (non-surgical cohort). To evaluate *KRAS* and *GNAS* mutation distribution and the performance of mutation analysis in cyst diagnosis, three groups of lesions were defined according to surgical pathology or EUS-FNA cytology and prolonged clinical follow-up: Group 1) Non-mucinous cysts (NMC), including neoplastic benign/inflammatory cysts (SCAs, lymphangiomas, and pseudocysts), and NETs; Group 2) Low-risk mucinous cysts (LRMC), including IPMNs and MCNs with LG; and Group 3) High-risk/malignant mucinous cysts (HRMC), including ADCs, IPMNs, and MCNs with HG. To understand its effect in decision making, a cyst classification according to mutational status information was compared with the diagnosis based on CEA and cytology.

EUS-FNA PCF Collection and Storage

In all patients undergoing EUS-FNA for evaluation of a pancreatic cyst, the PCF obtained is immediately separated into two samples. Sample A (0.5 mL) is centrifuged for cytospin preparation for cytological analysis, and the supernatant fluid is sent for CEA and amylase evaluation; sample B (with the remaining volume of PCF) is immediately put on ice and stored at − 80 °C in 0.25 mL aliquots, no more than 30 min after collection. A maximum volume of 0.25 mL was used for this molecular analysis.

Case Selection

From 266 patients undergoing EUS-FNA for pancreatic cyst evaluation between 2008 and 2014, 102 frozen PCF samples were obtained and stored at − 80 °C. For this study, we performed molecular analysis in samples of 52 patients who had more than 1 mL (four aliquots) of PCF stored. Clinical data, including cyst characteristics and treatment decision, have been prospectively collected and registered.

EUS Imaging

EUS still images of all case reports were reviewed and cystic morphology (size, thick septa, mural nodules, wall thickening, or mass) and main pancreatic duct features (dilatation > 10 mm or cyst communication) documented. Cysts were classified according to AGA Guidelines [22] by number of "high risk features" (Table 1). According to imaging, cysts were also classified into one of three groups: (1) NMC (cases suggesting SCAs or with no septa or nodules and features of pancreatitis); (2) LRMC (< 3 cm, no wall thickening, mural nodule, or mass); and (3) HRMC (> 3 cm, wall or septa thickening, mass, mural nodule, or dilation of the pancreatic duct > 10 mm).

Table 1 Demographics and clinical characteristics of the study population

Female gender, *n* (%) (*n* = 52)	35 (67.3%)
Mean age at EUS-FNA, year, mean ± SD (interval)	59.1 ± 14.8 (29–91)
Cyst location, *n* (%) (*n* = 52)	
Head	22 (42.3%)
Body	20 (38.5%)
Tail	9 (17.3%)
Multiple cyst locations	1 (1.9%)
Cyst size, cm, mean ± SD (interval)	3.9 ± 2.3 (1–10)
Cyst size > 3 cm, *n* (%)	29 (55.8%)
Cyst with nodule/mass, *n* (%)	18 (34.6%)
EUS imaging, *n* (%) (*n* = 52)[22]	
No high-risk features	13 (25%)
1 high-risk feature	29 (55.8%)
≥ 2 risk features	10 (19.2%)
PCF CEA, *n* (%) (*n* = 52)	
CEA < 192 ng/mL, *n* (%)	31 (59.6%)
CEA ≥ 192 ng/mL, *n* (%)	17 (32.7%)
No result available	4 (7.7%)
PCF cytology, *n* (%) (*n* = 52)	
Non-diagnostic	27 (51.9%)
Negative for malignancy	14 (26.9%)
Suspicious/malignant	10 (19.2%)
NET	1 (2%)
Treatment decision, *n* (%) (*n* = 52)	
Follow-up	34 (65.4%)
Surgery	11 (21.2%)
Endoscopic drainage	1 (1.9%)
Palliation (symptomatic or chemotherapy)	6 (11.5%)

EUS-FNA, endoscopic ultrasound with fine needle aspiration; high-risk features: cyst size ≥ 3 and solid component or thick wall or dilated Wirsung (> 10 mm); CEA, carcinoembryonic antigen; NET, neuroendocrine tumor; PCF, pancreatic cyst fluid; SD, standard deviation

PCF Analysis of CEA and Cytology

In all 52 patients, 0.5 mL of PCF was submitted for CEA determination. A level greater than 192 ng/mL prompted a classification of a mucinous cyst, and lower than 192 ng/mL of a non-mucinous cyst. Cytological analysis of PCF according to the Papanicolaou Society of Cytopathology Guidelines [12] classified the cysts in one of the three previously defined groups: atypical or malignant cells defined a cyst as a HRMC; mucinous benign epithelia without atypia or with low-grade atypia as a LRMC; and inflammatory cells, neoplastic benign non-mucinous cells, or other neoplastic cells (pancreatic neuroendocrine tumor—PanNET or solid pseudopapillary neoplasms—SPN) as a NMC. In patients with a non-diagnostic cytology, cyst classification was based on CEA, imaging, and long-term follow-up (at least 14 months) or a surgical pathology report.

Treatment Decision

The treatment decision was in accordance with the consensus guidelines of Sendai 2006 [13] revised in Fukuoka in 2012 [14]. High-risk cysts due to suspicious imaging (size > 3 cm and/or mass/mural nodule/thick wall) or a malignant cytology, HG epithelial atypia or suspicious cytology (atypical cells) in good surgical candidates, were referred for surgery. NETs were classified as a malignant NMC and referred for surgery. LRMC or benign NMC were kept on follow-up. Patients with invasive or locally advanced lesions and a positive/malignant cytology were offered palliation. One patient with a symptomatic pseudocyst had endoscopic drainage.

KRAS and *GNAS* Mutation Analysis

DNA was isolated from 250 µL of PCF using the Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (Norgen Biotek Corp., ON, Canada). Mutational analysis of *KRAS* (exons 2 and 3) and *GNAS* (exons 8 and 9) was performed by Sanger sequencing. The exons were PCR amplified in a standard PCR buffer (Invitrogen, Waltham, MA, USA) using specific primers. Sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the respective products analyzed on the ABI PRISM™ 3130 Genetic Analyzer, using the Sequencing Analysis software v3.4.1 (Applied Biosystems, Foster City, CA, USA). If no mutations were found, cysts were considered to represent wild-type *KRAS* and *GNAS*. In a few cases in which no DNA amplification was obtained for one of the exons, after several repeated readings and technical optimization, the result was considered non-informative.

Statistical Analysis

Descriptive data are expressed as mean ± SD. Fisher's exact test was used to assess differences between DNA-mutant and DNA-wild-type cysts for dichotomous variables. All tests were two-sided and statistical significance was defined as a p value < 0.05.

The sensitivity, specificity, positive and negative predictive value, and diagnostic accuracy of EUS imaging, CEA level, cytology, and *KRAS* and *GNAS* mutational status in PCF were evaluated for the diagnosis of mucinous cysts and high-risk mucinous/malignant cysts. Receiver operator curves were generated, and area under the curve (AUC) was calculated. Pancreatic cyst final diagnoses were based on surgical specimens or, in the non-surgical cohort, on PCF CEA, cytology and outcome combination after prolonged follow-up. Statistical analysis was performed using SPSS Statistics version 23.

Results

Demographics, Cystic Lesion Standard Analysis, and Clinical Decision

Between 2008 and 2014, 52 patients who underwent EUS-FNA for evaluation of a pancreatic cyst and who had at least 1 mL of PCF stored at − 80 °C had one aliquot of 0.25 mL retrieved for molecular analysis. The study population characteristics are displayed in Table 1, and the flowchart with clinical management is detailed in Fig. 1.

In our series, 35/52 patients were females, with a mean age of 59 years old, with cysts located predominantly in pancreatic head and body, and a mean size of 3.9 cm. Most were incidental findings, with 75% (39/52) of asymptomatic patients, 23% (12/52) with complaints of abdominal pain, and 2% (1/52) with vomiting. No patients presented with pancreatitis or jaundice.

After EUS-FNA, PCF was sent for CEA and cytology in all cases. A CEA determination was obtained in 92% (48/52) and a conclusive cytology in 48% (25/52) of PCFs.

In this series, 8/52 (15%) cysts were malignant, with seven malignant mucinous cysts and one NET. The diagnoses were either in the surgical specimen (one NET, one adenocarcinoma, one IPMN with invasive carcinoma) or

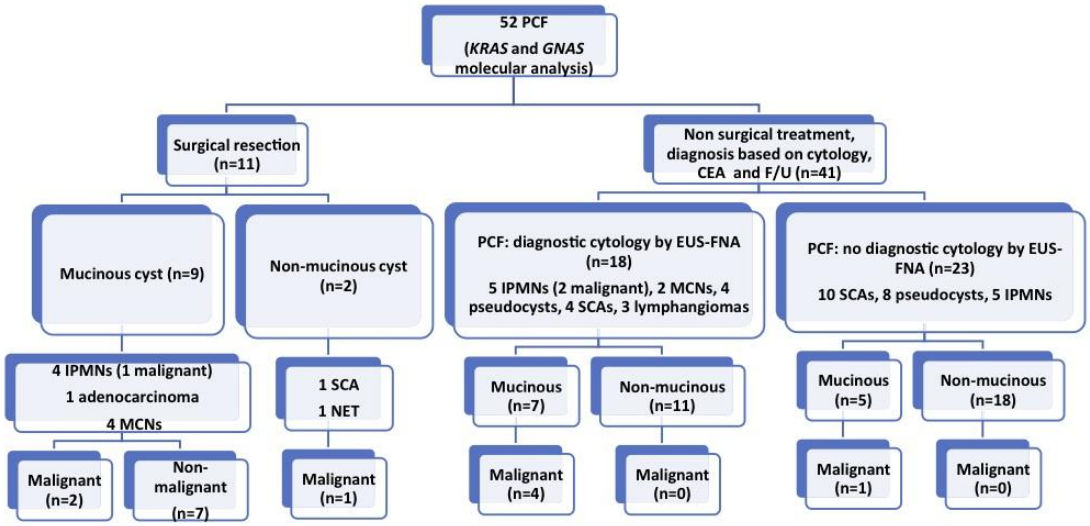

Fig. 1 Flowchart showing treatment decision after EUS-FNA. Surgical pathology or EUS-FNA (CEA ± cytology) and prolonged follow-up were used for final cyst classification as mucinous *vs* non-mucinous and malignant *vs* non-malignant (low-risk). *KRAS*, oncogene of Kirsten rat sarcoma virus; *GNAS*, gene of guanine nucleotide binding protein, alpha stimulating; F/U, follow-up; IPMNs, intraductal papillary mucinous neoplasms; MCN, mucinous cystic neoplasms; SCAs, serous cystadenomas; PCF, pancreatic cyst fluid; low-risk lesion means cytology with low-grade or intermediate-grade dysplasia; malignant stands for high-grade dysplasia, invasive carcinoma or NET

with disease progression and pancreatic cancer death on follow-up (four patients with a positive cytology and one with two consecutive inconclusive cytologic examinations). As shown in Fig. 1, benign cysts were diagnosed either in the surgical specimen (one SCA, three IPMNs, and four MCNs) or with characteristic imaging features and a positive EUS-FNA cytology and/or CEA, which prompted a diagnosis of IPMN, MCN, pseudocyst, SCA, or lymphangioma after a prolonged mean follow-up time of 57 ± 35 (14–156) months in the non-surgical series.

After clinical, imaging, and standard cystic fluid analysis, 31/52 lesions (60%) were classified as non-mucinous and 21/52 lesions (40%) as mucinous. Clinical decision, after EUS-FNA, was surgical resection in 11 (21%) patients, palliation in six (12%), endoscopic drainage in one (2%), and 34 with low-risk cysts were referred for follow-up. The follow-up was performed with EUS in 6 months in cysts larger than 3 cm, with mural nodules, thickened wall, or a dilated pancreatic duct (5–9 mm). In cysts without worrisome features, follow-up was performed by MRI every year during the first 2 years and then every other year in surgically fit patients. In patients with a diagnostic cytology (malignant or atypical) or with high-risk stigmata (jaundice, pancreatic duct dilation over 10 mm, or an enhancing solid component in the cyst), surgical resection was recommended and follow-up would stop. If the pancreatic malignant lesion was found to be unresectable, palliation with chemotherapy or pain medication was recommended.

In the 11 resected cysts, there were three malignant cysts (one ADC, one IPMN-IC, one NET), seven mucinous low-risk (three IPMN-LG, four MCN-LG), and one benign cyst (one SCA). The mean follow-up time in the whole series of 52 patients was 45 ± 36 (3–40) months but was longer in the 34 patients on follow-up, with a mean time of 57 ± 35 (14–156) months. Of these 34 patients, five (10%) with benign/inflammatory cysts stopped follow-up (SCNs, lymphangiomas) or were lost for follow-up, and 29 (56%) are still on follow-up with no morphological changes in cystic lesions. In this series, five (9.6%) patients died (three of pancreatic cancer, one from other neoplasia, and one of pancreatic surgery complications).

Molecular Analysis

KRAS and *GNAS* mutational analysis was performed in the 52 patients after clinical decision and did not interfere with it. Table 2 presents clinical, imagiological, and PCF analysis, clinical decision, and definitive diagnosis in nine cysts with *KRAS/GNAS* mutations. Although 5/9 patients with *KRAS*-mutated cysts had an invasive adenocarcinoma, the remaining four patients had low-risk mucinous cysts. All five patients with malignancy were symptomatic, PCF had CEA > 192 ng/mL, cytology was positive for malignancy in 4/5 patients, and EUS high-risk features were present in 4/5 patients as well. There were only two samples with a *GNAS* mutation and in both a *KRAS* mutation was also present, one in an IPMN-LG and the other in an unresectable adenocarcinoma referred for chemotherapy. In 5/52 samples, no DNA amplification occurred in at least one of the exons analyzed and the test was considered non-informative (two presumed pseudocysts, two carcinomas, and one IPMN).

Table 3 summarizes patients' and cysts' characteristics in the 47 patients with informative KRAS mutational analysis. There was no gender preponderance in patients harboring cysts with *KRAS* mutations, but these were significantly older (69.2 vs 56.5 years, $p = 0.01$). No significant differences were found in cyst size or location, but mutations were more frequent in mucinous cysts (CEA > 192 ng/mL, $p = 0.001$). In lesions with EUS high-risk features, no significant differences were detected ($p = 0.08$). In the ten patients of this subgroup who were submitted to surgery (two NMC, six LRMC, and two HRMC), no association was found between *KRAS* mutation and surgical pathology diagnosis.

To understand the discriminant power of molecular analysis between the three categories of cystic lesions previously defined (NMC, LRMC, and HRMC), a sub-analysis of 29 patients with a definitive diagnosis, either with a surgical pathology specimen or a diagnostic EUS-FNA cytology and prolonged follow-up, was performed. Table 4 shows that *KRAS/GNAS* mutations were more frequent in mucinous lesions, both LRMC and HRMC, as compared with NMC, which were all *KRAS* WT ($p = 0.045$).

Table 5 shows the performance of the several tests, alone and in combination, to diagnose mucinous cysts. CEA has the highest sensitivity and specificity and was the best test (AUC = 0.93). Molecular analysis did not perform as well (AUC = 0.72), with a low sensitivity (S = 50%) but a specificity of 100%. However, in clinical practice, the most relevant distinction is between low-risk and high-risk mucinous cysts, as only the latter should have surgical resection. Results for high-risk mucinous cysts are shown in Table 6. EUS imaging has the best diagnostic performance (AUC = 0.86), and the combination of EUS imaging and cytology was more accurate than full cystic fluid analysis with CEA, cytology, and mutational analysis associated.

Molecular Testing Information on Clinical Decision Making Considering the Whole Series

When evaluating the diagnostic advantage of molecular analysis over conventional methods in discriminating between HRMC, LRMC, and NMC, we observed that the nine *KRAS/GNAS* mutations occurred in both LRMC and HRMC. These were diagnosed as such using conventional methods, except for one *KRAS* mutation observed in a patient with a presumed pseudocyst. Table 7 summarizes this information.

Table 2 Detailed clinical, imagiological, biochemical, cytologic features and final diagnosis of 9 mutated *KRAS/GNAS* cysts

Patient	Gender	Age	Clinical symptoms	Cyst size (cm)	Cyst location	Ductal dilation	Mural nodule or mass	CEA (ng/mL)	Malignant cytopathology	KRAS mutation	GNAS mutation	Clinical decision	Diagnostic surgical pathology
1	F	81	Asymptomatic	1.9	Body	No	Absent	300,754	Neoplastic: Benign (IPMN-LG)	Exon 2 G12A	Wild type	F/U (84 months)	No (EUS-FNA and F/U)
2	F	64	Pain	1	Body	No	Present	11,764	Positive (AdenoCa)	Exon 2 G12D	Wild type	Chemotherapy	No (EUS-FNA and F/U)
3	F	63	Pain	6	Body	No	Present	3802	Positive (AdenoCa)	Exon 2 G12D	Exon 8 R201H	Chemotherapy	No (EUS-FNA and F/U)
4	M	75	Pain	2	Head	No	Absent	10,003	Positive (AdenoCa)	Exon 2 G12D	Wild type	Surgery	AdenoCA in IPMN
5	M	62	Asymptomatic	2.1	Tail	No	Absent	125	Non-diagnostic	Exon 2 G12D	Wild type	F/U (48 months)	No (EUS-FNA and F/U)
6	M	65	Asymptomatic	3	Body	No	Absent	249	Non-diagnostic	Exon 2 G12R	Exon 8 R201H	Surgery	IPMN-LG
7	M	80	Pain	2.8	Tail	No	Present	150,490	Positive (AdenoCa)	Exon 2 G12 V	Wild type	Chemotherapy	No (EUS-FNA and F/U)
8	F	78	Pain	5	Head	Yes	Present	191,437	LG	Exon 3 E61H	Wild type	Palliation	No (EUS-FNA and F/U)
9	F	60	Asymptomatic	3.7	Tail	No	Present	1515	Atypical cells	Exon 3 E61H	Wild type	Surgery	MCN LGD

F—female, M—male, F/U—follow-up

Table 3 Clinics and characteristics of *KRAS* wild-type and *KRAS*-mutant cystic lesions

Patient and cyst characteristics ($n=47$[a])	*KRAS* wild-type	*KRAS* mutant	*p* value
Gender, *n*			
Female	27	5	0.438
Male	11	4	
Age (year)			
Mean ± SD	56.5 ± 14.6	69.2 ± 7.8	0.016
Cyst size (cm)			
Mean ± SD	4.1 ± 2.3	2.9 ± 1.7	0.171
Cyst location, *n*			
Head	17	3	
Body	14	3	
Tail	6	3	
Multiple locations	1	0	0.647
EUS diagnosis			
Non-mucinous	25	4	
Low-risk mucinous	12	3	
High-risk mucinous	1	2	0.085
CEA ($n=43$)			
CEA < 192 ng/mL	28	1	
CEA > 192 ng/mL	7	7	0.001
EUS-FNA cytological diagnosis			
Non-diagnostic	22	3	
Negative for malignancy/neoplastic benign	10	1	
Atypical cells/positive for malignancy	5	5	0.011
Surgical Specimens ($n=10$)			
MCN—LG and IPMN-LG	4	2	
Adenocarcinoma/IPMN—IC	1	1	
NET	1	0	
SCA	1	0	0.374

SD, standard deviation; IC, invasive carcinoma; LG, low grade

[a]Only the 47 PCF with conclusive mutational analysis in all the four exons of *KRAS* and *GNAS* studied were considered for analysis. In five samples, no DNA amplification occurred in at least one of the exons analyzed and mutational analysis was considered non-diagnostic

Table 4 Distributions of *KRAS* mutations in three groups of cystic lesions (NMC, LRMC, and HRMC) in the 29 patients with a conclusive diagnosis obtained by EUS-FNA cytology and a prolonged follow-up or surgical pathology

$N=29$ patients with a diagnostic EUS-FNA cytology and F/U or a pathology diagnosis	Mutant *KRAS/GNAS*	Wild type	Non-diagnostic
NMC (SCA, pseudocyst, NET, lymphangioma) ($n=12$)	0	11	1
LRMC ($n=10$)	3	6	1
HRMC ($n=7$)	5	2	0

F/U, follow-up, NMC, non-mucinous cysts; LRMC, low-risk mucinous cyst; HRMC, high-risk/malignant mucinous cyst

Analysis of molecular testing according to clinical decision in the 52 patients shows that 11 underwent surgery (two with *KRAS* and one with *KRAS/GNAS* mutation), six had palliation/chemotherapy (three with *KRAS* and one with *KRAS/GNAS* mutation), one was submitted to endoscopic drainage, and 34 referred for follow-up, with no change in cyst characteristics or size after a mean time of 57 ± 35 (14–156) months, including two patients with *KRAS* mutations in a LRMC and in a presumed pseudocyst, both stable after 84 and 48 months of follow-up, respectively.

Table 8 shows cyst classification modification according to molecular diagnosis if *KRAS* mutation is considered

Table 5 Performance characteristics of EUS imaging, cystic fluid CEA, cytology, and molecular analysis for mucinous cystic lesion identification

Mucinous cysts = (10 LRMC + 7 HRMC)/29	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95%CI)	Accuracy (95% CI)	Area under the ROC (CI)
EUS: imaging features	53 (28–77)	83 (52–98)	82 (54–95)	56 (42–67)	66 (44–82)	0.68 (0.48–0.88)
EUS-FNA: CEA > 192 ng/mL	93 (66–100)	92 (62–100)	93 (66–99)	92 (63–99)	92 (75–99)	0.93 (0.83–1)
EUS-FNA: cytology	56 (28–77)	83 (52–98)	82 (54–95)	56 (42–69)	66 (46–82)	0.54 (0.31–0.77)
EUS-FNA: mutational analysis (*KRAS/GNAS*)	50 (25–75)	100 (72–100)	100	58 (46–69)	70 (50–86)	0.72 (0.52–0.92)
EUS-FNA: CEA ↑ or cytology + or mutational analysis +	94 (67–100)	77 (46–95)	83 (65–93)	91 (54–99)	86 (68–96)	0.84 (0.68–1)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; ROC, receiver-operating characteristics

Table 6 Performance characteristics of EUS imaging, cystic fluid CEA, cytology, and molecular analysis for HRMC identification

HRMC = 7/29	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)	Area under the ROC (CI)
EUS: imaging features	88 (47–100)	81 (58–95)	64 (41–81)	94 (73–99)	83 (64–94)	0.86 (0.68–1)
EUS-FNA: CEA > 192 ng/mL	86 (42–100)	58 (34–80)	43 (29–58)	92 (63–99)	65 (44–83)	0.65 (0.44–0.86)
EUS-FNA: cytology	67 (30–93)	90 (68–99)	75 (43–92)	86 (70–94)	83 (64–94)	0.79 (0.57–1)
EUS-FNA: mutational analysis (*KRAS/GNAS*)	63 (25–91)	82 (60–97)	63 (34–84)	83 (68–93)	78 (58–91)	0.73 (0.48–0.93)
EUS-FNA: CEA ↑ or cytology + or mutational analysis +	88 (47–100)	57 (34–78)	44 (31–58)	92 (65–99)	66 (46–82)	0.79 (0.62–0.95)
EUS-FNA: Imaging features or cytology +	100 (63–100)	71(48–89)	57 (40–72)	100	79 (60–92)	0.86 (0.72–0.99)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; ROC, receiver-operating characteristics

Table 7 Molecular analysis results in low-risk mucinous, high-risk mucinous and non-mucinous cysts as classified after clinical, imaging, and CEA and cytology obtained by EUS-FNA

52 cysts	LRMC ($n = 14$)	HRMC ($n = 7$)	NMC ($n = 31$)
KRAS wild type	9	2	27
KRAS mutation	3	5	1
KRAS non-diagnostic	2	0	3
Total	14	7	31

LRMC, low-risk mucinous cyst; HRMC, high-risk mucinous/malignant cyst; NMC, non-mucinous cyst

synonymous with mucinous. In the LRMC, 6/12 would have been classified as NMC according to *KRAS* status, which would be false in all cases. In contrast, 3/8 HRMC would be classified as "non-malignant," but this would be correct in 1/3 lesions only. This was in fact a false positive cytological diagnosis of malignancy in the case of a 56-year-old female with a 2.9-cm cyst, CEA in PCF lower than 0.5 ng/mL, who was referred for surgery, with a final surgical pathology diagnosis of a SCA. Also, one change would occur in a lesion classified as a pseudocyst which, after molecular testing, was correctly re-classified as a LRMC. This cyst was diagnosed in a 62-year-old male, having a stable size of 2.1 cm after 48 months of follow-up, and EUS-FNA with a CEA = 125 ng/mL with a non-diagnostic cytology. It would be classified as a pseudocyst with standard PCF analysis, but is probably an IPMN with the *KRAS* mutation detected, with added value in cyst classification, as IPMNs can be under-diagnosed with a CEA cutoff level of 192 ng/mL.

Table 8 Effect of molecular analysis in cyst re-classification after conventional classification using clinical, imaging, and PCF CEA and cytology obtained by EUS-FNA

AFTER EUS-FNA ($N=52$)	KRAS non-diagnostic *n/N* (%)	Supported n/N (%)	Changed *n/N*%	Correct Change *n/N* (%)
Mucinous cysts ($N=21$)	2/21(9.5%)	7/21 (33.3%)	12/21 (57.1%)[a]	1/12(8.3%)
NMC ($N=31$)	3/31 (9.7%)	27/31 (87.1%)	1/31 (3.2%)[b]	1/1 (100%)

NMC, non-mucinous cyst

[a]One ADC, four MCN, one IPMN, and one SCA (with a preoperative false positive cytology) operated and five cysts on follow-up, with CEA ↑ and cytology with inflammatory cells (2) and non-diagnostic (2) and benign cells (1); [b]1 pseudocyst (CEA = 125 and a non-diagnostic cytology)

Overall, only 2/10 (20%) of changes in cysts re-allocation due to mutational analysis are valuable for improved cyst classification, increasing LRMC diagnosis, previously classified as inflammatory and not confirming a HRMC with a false positive malignant cytology.

Discussion

In this cohort of patients with pancreatic cysts followed prospectively over a mean period of almost 5 years and managed according to published guidelines, we observed that *KRAS* and *GNAS* mutations in PCF added very little when compared to standard clinical examinations—EUS-FNA with cytology and CEA. Molecular testing was able to distinguish between mucinous and non-mucinous lesions (AUC = 0.72), although values of CEA > 192 ng/mL were the most accurate test to identify mucinous lesions (AUC = 0.93). Regarding the most clinically significant, high-risk mucinous/malignant lesions, EUS imaging alone or combined with a diagnostic cytology was more accurate than molecular markers for diagnosis (AUC = 0.86 vs 0.73).

Expanded use of computed tomography and magnetic resonance resulted in increased detection of pancreatic cysts, some of which are believed to be precursors of pancreatic adenocarcinoma. Mucinous cysts have the potential to progress to malignancy [6], and current guidelines recommend surgical resection in "high-risk cysts" [14]. IPMNs can progress from low-, to intermediate-, to high-grade dysplasia, and ultimately to invasive carcinoma [8]. It is recommended that only IPMNs with high-grade epithelial atypia or an associated invasive carcinoma should undergo resection, while IPMNs with low-grade epithelial atypia can undergo surveillance.

However, cross-sectional imaging is not accurate in distinguishing different cyst types [3], and cystic fluid analysis with CEA and cytology is imperfect for cyst evaluation [4–7]. In our series, CEA was measurable in 90% of the samples but cytology was informative in only 48% of cases. The accuracy of cytology from EUS-FNA samples ranges from 54 to 97%, but may be lower in smaller cysts [6]. In a recently published meta-analysis, CEA was found to have a sensitivity of 63% and a specificity of 88% to identify mucinous cystic tumors [5]. As cystic fluid CEA and cytology are considered investigational in some of the current guidelines [14], in clinical practice management of pancreatic cysts remains difficult and highly individualized.

The high frequency of pancreatic cyst detection, the significant efforts for cyst follow-up, and the significant morbidity and mortality of pancreatic surgery for a possible benign disease make an accurate pancreatic cyst classification critical in clinical practice.

Molecular analysis of PCF is useful for pancreatic cyst classification and advanced neoplasia detection [15–19], but its performance in clinical practice remains unclear. Some important studies [15], which include large numbers of surgical patients and a large panel of molecular markers, do not mimic a clinical practice scenario, in which the physician facing a patient with a pancreatic cyst recommends surveillance or surgery using clinical, imagiological, cytological, and biochemical information. Our study adheres to current practice and patients were all managed according to Sendai [13] or Fukuoka [14] guidelines. In agreement with more recent and restrictive guidelines [20], few patients had surgery (11/52) and the majority (34/52) are on follow-up for a mean period of almost 5 years, all for more than 14 months. Of note, cystic fluid was obtained from EUS-FNA and not during surgery, as in some other series [15]. Most of these doubtful lesions are small, and aspirated volume is usually scant. In contrast to studies including only surgical patients [15, 16], it is important to examine what happens to patients with *KRAS*-positive lesions who are not resected. In the present series, 2/9 patients harboring *KRAS*-mutated lesions are alive and stable after 84 and 48 months of follow-up, respectively.

Our study is in agreement with earlier publications [21], reporting that *KRAS* mutations were highly specific to identify mucinous lesions. *KRAS* mutation had 100% specificity, an acceptable discriminative power (AUC = 0.72), but a low sensitivity (50%).

In a previous publication with resected cysts [15], *KRAS* was the most prevalent altered gene in the cyst fluid samples from IPMNs (78%). In another large prospective study of DNA-molecular testing in PCF prior to surgery, the

limitations of Sanger sequencing compared to Next-Generation Sequencing (NGS) in pancreatic cyst evaluation [18] were evident. The lower prevalence of *KRAS* mutations found in the present study (17%) may be related to the cyst types in our series (mostly non-surgical), low PCF volume, and the use of Sanger, which has lower sensitivity than NGS. However, we should recall that increasing technique sensitivity may increase the number of false positives, possibly increasing unnecessary surgeries. In fact, whereas a pancreatic mucinous lesion was previously considered worrisome, we now know that most of these lesions will not progress to malignancy [22] and the critical issue for patient care is diagnosing high-risk mucinous/malignant mucinous lesions. In our study, the most accurate tests to diagnose these lesions were EUS-imaging findings associated whenever possible with cytologic samples. The added benefit of molecular markers in the present series was marginal—only 2/10 patients had a correct re-classification of their cystic lesions after molecular analysis, which would not affect clinical decision in either case.

In our study, *GNAS* mutation did not improve diagnosis. In fact, only two samples had a *GNAS* mutation, and in both of those patients, a concomitant *KRAS* was present, which leads us to conclude that in contrast to previous series, *GNAS* mutations are not sensitive to diagnose IPMNs.

One additional issue is that some malignant cysts, such as NETs, are not mucinous. In these lesions, CEA level is low, *KRAS* is not usually mutated, and cytology is fundamental for decision making. This further reinforces the belief that a combined or sequential analysis strategy must be the rule in pancreatic cysts. It makes sense to use the most sensitive method of diagnosis in young and healthy patients and to obtain more specific markers in older patients with comorbidities. In fact, in our surgical cohort of 11 patients, one surgery could have been avoided (one SCA) and seven surgeries, in low-risk mucinous cysts, at least delayed.

This study has several limitations. First, only 11/52 patients had surgery with an available surgical specimen. However, in the additional 18 patients with EUS-FNA cytology specimens, the extended period of observation allows us to assume the low-risk nature of the lesions. Second, as the samples selected had a stored volume over 1 mL, there may be a selection bias for larger cysts, but these are also those that raise more management problems. Finally, we may have an underdiagnosis of mucinous cysts due to the cutoff CEA level of 192 ng/mL. In our series, 32 cysts had a CEA < 192 ng/mL with a mean CEA level of 26.8 ± 33 ng/mL (0.5–125), of which 13 had CEA ≤ 5 ng/mL. We had only one *KRAS* mutation among these 32 cysts, a cyst with CEA = 125 ng/mL and a non-diagnostic cytology, with one additional mucinous cyst diagnosis.

In summary, our results do not support *KRAS* and/or *GNAS* mutational analysis using Sanger sequencing in pancreatic cysts. Although we found that *KRAS* mutations occur predominantly in mucinous and malignant cysts, CEA level for low-risk mucinous cysts and combined imaging and cytology for high-risk mucinous/malignant cysts were more accurate. As of today, the difficult decision the clinician faces when finding a pancreatic cyst is what criteria to use to differentiate between low- and high-risk mucinous lesions. In the present study, we have patients with non-operated cysts showing no changes after a mean period of 47 months and in some of these lesions a *KRAS* mutation was found. As imaging and cytological analysis performed better in discriminating between low- and high-risk mucinous lesions, we conclude that the final contribution of molecular diagnosis in real-life practice seems very low or null. Mutational analysis using Sanger sequencing might be a limitation of this study, but we must recognize that cutting edge technology such as NGS is not readily available in most clinical centers and its cost-efficiency in decision making must be validated before it can be recommended in current practice. The sequential use of mutational analysis may be recommended to diagnose mucinous cysts in young and well-fit patients after a non-diagnostic cytology and CEA < 192 ng/mL that would otherwise stop follow-up due to a false negative diagnosis of mucinous cyst type.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Excellent Accuracy of Glucose Level in Cystic Fluid for Diagnosis of Pancreatic Mucinous Cysts

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Abstract

Background CEA in pancreatic cystic fluid (PCF) is standard for mucinous cysts diagnosis. Glucose is an alternative, but its accuracy remains poorly described.

Aims To evaluate PCF glucose using a glucometer and compare its accuracy with CEA for mucinous cysts diagnosis.

Materials and Methods In frozen PCF obtained by EUS-FNA, glucose was evaluated using a glucometer. CEA and cytology were available as standard of care. The accuracy of glucose and CEA was calculated using receiver operator (ROC) curves. Definitive diagnoses were surgical or clinicopathological.

Results We evaluated 82 patients with a mean age of 61.3 ± 14.8 years (25–91), predominantly (59%) females. Diagnoses included 17 serous cystadenomas, five pseudocysts, 20 intraductal papillary mucinous neoplasms, three mucinous cystic neoplasms, five adenocarcinomas, four neuroendocrine tumors, two other types, 26 non-defined. The median glucose levels (interquartile range) were 19 mg/dL (19–19) in mucinous and 105 mg/dL (96–127) in non-mucinous cysts ($p < 0.0001$). The median CEA level was 741 ng/mL (165–28,567) in mucinous and 9 ng/mL (5–19) in non-mucinous cysts ($p < 0.0001$). For mucinous cyst diagnosis, a CEA > 192 ng/mL had a sensitivity of 72% (95% CI 51–88) and a specificity of 96% (95% CI 82–100), and ROC analysis showed an area under the curve (AUC) of 0.842 (95% CI 0.726–0.959), while glucose < 50 mg/dL had a sensitivity of 89% (95% CI 72–98), a specificity of 86% (95% CI 67–96), and an AUC of 0.86 (95% CI 0.748–0.973). Pseudocysts presented low glucose, identically to mucinous cysts, with CEA allowing differential diagnosis.

Conclusion Glucose measured by a glucometer is accurate for mucinous cyst diagnosis, with significantly higher levels in non-mucinous cysts, except pseudocysts.

Keywords Glucose · CEA · Pancreatic cyst · EUS-FNA · IPMN · MCN

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Introduction

The widespread use and technical advances of abdominal imaging allied with population ageing have led to an increase in the detection of asymptomatic pancreatic cystic lesions (PCLs) [1]. PCLs encompass a wide spectrum of diagnoses that range from benign/inflammatory lesions [e.g., serous cystadenomas (SCAs), pseudocysts] to premalignant [intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs)], and malignant cysts [cystic adenocarcinomas (ADCs), cystic neuroendocrine tumors (NETs), acinar cell carcinomas, etc.]. The differential diagnosis between PCLs is important, since the clinical approach differs, with surgery required for high-risk and malignant cysts, surveillance for premalignant lesions, and no follow-up recommended for benign cysts. Currently, the diagnosis of these lesions includes pancreatic cystic fluid (PCF) analysis obtained by endoscopic ultrasound with fine-needle aspiration (EUS-FNA) [2, 3].

Pancreatic mucinous cysts are precursors of pancreatic cancer including MCNs and the most frequent IPMNs and the differential from other PCLs is not trivial [4]. Their malignant potential in surgical series encompasses 10% of noninvasive and 13% of invasive carcinomas [5].

The preoperative diagnosis of mucinous cysts is based on an elevated carcinoembryonic antigen (CEA) level in PCF, and several pitfalls have been reported with this biomarker. First, the cutoff levels vary between laboratories [6–8], limiting its reproducibility and making the interpretation of CEA results difficult. Second, a significant volume of PCF [at least 200 μL] is required for CEA analysis, precluding its measurement when PCF is scant. Finally, the currently used CEA level (> 192 ng/mL) has limited sensitivity for mucinous cyst diagnosis, with considerable overlap in CEA levels between mucinous and non-mucinous cysts [7]. To overcome these issues and increase the diagnostic yield, additional biomarkers have been analyzed in PCF, but these increase the complexity and costs considerably [9–12].

An exploratory metabolomics study revealed that low levels of glucose in PCF had diagnostic value for identifying mucinous cysts [13]. This finding was later confirmed in two clinical studies with laboratorial and/or glucometer measurement of glucose in PCF [14, 15]. However, both studies used aspirates mostly from surgically resected lesions, a situation that differs from real-life practice, in which we need a preoperative diagnosis.

The goal of our study is to evaluate the accuracy of glucose level measured with a glucometer, in PCF collected preoperatively by EUS-FNA, for diagnosis of mucinous cysts, and to compare its accuracy with that of laboratorially obtained CEA level.

Patients and Methods

Sample Acquisition and Case Selection

This longitudinal cohort study was approved by the Ethics Committee and Institutional Scientific Board (UIC/1225).

We selected PCF samples from our Endoscopic Ultrasound Registry and PCF biorepository, in which clinical data, EUS morphology of the cyst, PCF analysis including CEA and amylase, clinical decision, and follow-up, were prospectively collected and recorded. All patients gave informed consent for EUS-FNA, standard PCF analysis, and remnant volume storage. Immediately after FNA, the PCF was collected into a sterile dry tube that was put on ice and sent to the cytology laboratory, to be centrifuged for 10 min at 2000 g, for cytospin preparation for cytology. There supernatant was separated into two samples: Sample A (0.5 mL) was sent to laboratory for routine analysis with CEA, amylase, and cytology in cytospin (performed by experienced cytopathologists); Sample B (remnant PCF) was stored, no more than 30 min after collection, in 0.25 mL aliquots at − 80 °C until further analysis, in order to minimize thawing and refreezing cycles and stabilize PCF analytes. The amount of PCF stored per patient was variable, according to remnant after standard analysis.

The study cohort consisted of a selected group of 82 patients with pancreatic cysts. The main criterion for patient selection was having more than 1 mL of frozen PCF, in either surgical (definitive surgical pathology) or clinicopathological cohorts [EUS-FNA with PCF analysis, with CEA ± diagnostic cytology, and documented stability after prolonged (> 24 months) surveillance].

Additionally, in some patients, PCF had been sent for laboratory evaluation of glucose ($n = 19$) and the same type of glucometer "on-site" evaluation at time of EUS-FNA ($n = 7$). These results were retrospectively retrieved from electronic medical records, with the purpose of evaluating the reproducibility of glucose measurement in the frozen samples of PCF.

Standard EUS Imaging and PCF Analysis with CEA and Cytology

EUS findings, including cystic size, location, morphology (thick septa, mural nodules, wall thickening, or mass), and main pancreatic duct features (dilatation > 10 mm or cyst communication), were retrieved from our database of prospective collected data. In all 82 samples, PCF was evaluated for CEA (Architect, Abbott; chemiluminescent immunoassay) and amylase (Architect, Abbott; kinetic colorimetric method), with CEA (ng/mL) and amylase (UI/L) values available for 78/82 PCLs.

A CEA level greater than 192 ng/mL prompted a classification of a mucinous cyst and lower than 192 ng/mL of a non-mucinous cyst. Cytological analysis of PCF subclassified the cysts into different types, and (in the current study) for acellular samples, the cyst was classified as indeterminate. The reference standard for glucose accuracy analysis was the histopathology in the surgical cohort and a definitive cytology in the clinical cohort.

After undergoing EUS-FNA, patients were referred for surgery (surgical cohort) or surveillance, palliation, or endoscopic drainage (non-surgical cohort).

Glucose Assay

For this study, we evaluated glucose level with a standard glucometer, the Verio One Touch IQ glucometer (LifeScan Europe, Switzerland) [16] that was also used in previous studies [14, 15], using an aliquot of frozen PCF. To minimize variation in analytes, all samples were processed within 30 min of thawing, and 2 µL of cystic fluid was analyzed, by pipetting the PCF onto the side of the testing strip. The OneTouch glucometer measures glucose levels between 20 mg/dL and 600 mg/dL, requiring only 0.4 µL of sample. For numerical analysis, we registered glucose readings < 20 mg/dL as 19 mg/dL. The person performing the measurements was blinded to the final diagnosis. To ensure reproducibility of our measurements, we compared the glucose levels obtained from the frozen samples of PCF, with the glucose levels obtained in fresh PCF at the time of EUS-FNA evaluated in the laboratory (Architect, Abbott; test range of 5–800 mg/dL) ($n = 19$) and/or "on site" using the same glucometer (test range of 20–600 mg/dL) ($n = 7$).

Statistical Analysis

Descriptive data are expressed as mean ± SD, median, and interquartile range. To determine differences between cyst types, the Mann–Whitney U test was used for continuous variables and the Fisher's exact test for categorical variables.

Receiver operating characteristic (ROC) curves were generated, and area under the curve (AUC) was calculated to differentiate mucinous from non-mucinous cysts using CEA, glucose, and the combination of CEA with glucose. Correlations between glucose in frozen PCF and CEA, and between glucose in the frozen PCF and fresh PCF using the glucometer or the laboratory assay, were measured using the Spearman's rank correlation coefficient. Statistical significance was defined as a p value < 0.05.

Statistical analysis was performed using SPSS Statistics version 24 (Armonk, NY).

Results

Demographics and Cyst Characteristics

We included 82 patients with frozen PCF samples, of which 78 had prior CEA and amylase measurements. All samples were assessed for glucose levels (supplementary Table 1).

The patient population was composed predominantly of females (59%); the mean age was 61.3 ± 14.8 years (25–91). Pancreatic cysts were located mainly in the head (53%), body (27%), tail (18%), and multiple locations (2%). The mean size of the cysts was 38.5 ± 20.4 mm (8–100), and 67.1% (55/82) of PCLs were larger than 30 mm.

The cyst types included in our study are represented in the flowchart of Fig. 1. There were 56/82 lesions with surgical ($n = 12$) or EUS-FNA conclusive cytology ($n = 44$), including 17 SCAs, five pseudocysts, 20 IPMNs, three MCNs, five ADCs, four NETs, and two other very rare cyst types (one lymphangioma and one acinar cell carcinoma). There were 26 lesions, in which, after PCF analysis, the CEA level obtained, simultaneously, with a non-conclusive cytology, precluded the possibility of cyst-type classification. The descriptive data of these 26 samples were analyzed but not included in diagnostic accuracy analysis.

By means of a cutoff value of 192 mg/dL for CEA level, PCLs with conclusive cytopathological diagnoses ($n = 56$) were further classified as: (a) mucinous cysts ($n = 28$), including 20 IPMNs, three MCNs, and five ADCs; and (b) non-mucinous cysts ($n = 28$), including 17 SCAs, five pseudocysts, four NETs, and two other cyst types.

Table 1 shows clinical, endosonographic, and biochemical PCF data of all 82 patients with frozen samples evaluated for glucose level using the glucometer. Patients' gender, cyst size, and cyst location did not differ between mucinous and non-mucinous PCLs, but patients with mucinous cysts were older ($p = 0.018$), had symptomatic cysts more frequently ($p = 0.032$), and presented mural nodules more often ($p = 0.020$).

Concerning PCF analysis, mucinous cysts had higher amylase and CEA levels ($p = 0.021$ and $p < 0.0001$, respectively) and lower glucose ($p < 0.0001$), but no significant difference was detected in cytological diagnosis ($p = 0.822$) (Table 1).

CEA and Glucose Assays

Table 2a shows CEA and glucose levels for mucinous and non-mucinous lesions in which we included only the 56 patients with a definitive pathological or

Table 1 Demographic and cyst characteristics in mucinous versus non-mucinous cysts

	Mucinous ($n=38$)	Non-mucinous ($n=44$)	p value
Female, n (%)	20 (52.6%)	28 (63.6%)	0.372
Mean age ± SD (range)	65.3 ± 13.2 (33–81)	57.3 ± 15.4 (25–91)	0.018
Symptoms[a], n (%)	10 (26.3%)	1 (2.3%)	0.032
Cyst location (head, body, tail, multiple)	20/9/8/1	23/13/7/1	0.873
Cyst size (mm) mean ± SD (range)	36.7 ± 19.2 (11–100)	40.1 ± 21.5 (16–100)	0.533
Septa, n (%)	23 (60.5%)	30 (68.2%)	0.496
Nodule, n (%)	14 (36.8%)	6 (14%)	0.020
Adenopathy, n (%)	3 (7.9%)	0 (0%)	0.095
Amylase (U/L) median (IQR)	3986 (141–12,689)	124 (47–2174)	0.021
CEA (ng/mL) median (IQR)	525.5 (128–7391)	9 (5–20.5)	0.000
Glucose[b] (mg/dL) median (IQR)	19 (14.3–25)	99 (59–123.3)	0.000
Glucometer reading error	9 (23.7%)	0 (0%)	0.001
Conclusive cytology, n (%)	21 (58%)	27 (61%)	0.822

IQR interquartile range

[a]Pain, weight loss, vomiting

[b]Glucometer assay performed in frozen PCF samples

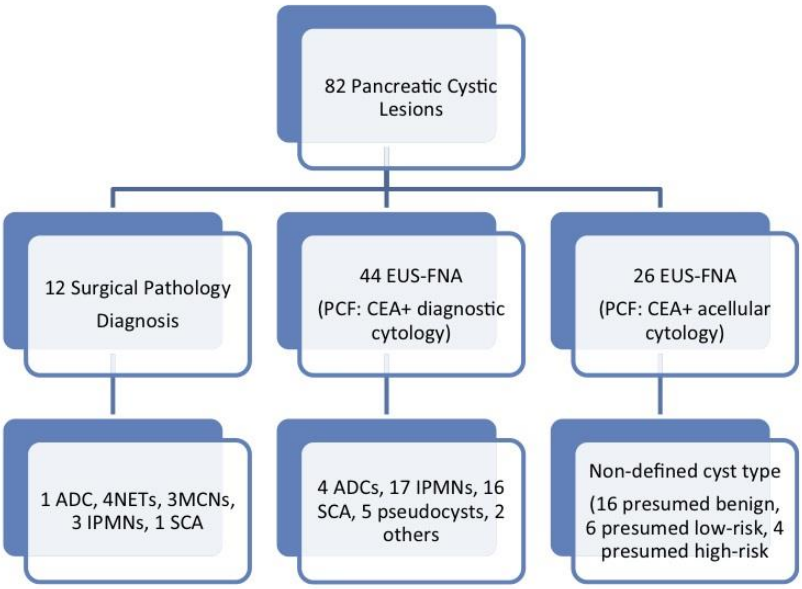

Fig. 1 Flowchart with the diagnoses of 82 pancreatic cystic lesions, after surgical pathology or EUS-FNA (CEA ± cytology) and prolonged follow-up. *PCF* pancreatic cyst fluid, *ADCs* adenocarcinomas, *NETs* neuroendocrine tumors, *MCNs* mucinous cystic neoplasms, *IPMNs* intraductal papillary mucinous neoplasms, *SCAs* serous cystadenomas; low-risk lesion stands for CEA > 192 mg/dL and no imaging worrisome features (neither nodule, mass, nor Wirsung dilatation); high-risk lesion stands for imaging worrisome feature (mural nodule, mass, or Wirsung > 5 mm) independently of CEA level

cytological diagnosis. Median CEA was 741 ng/mL (IQR: 165–28566.5) for mucinous and 9 ng/mL (IQR: 5–18.5) for non-mucinous cysts ($p < 0.0001$). Median glucose was < 20 mg/dL [(numerical analysis: 19 mg/dL (IQR: 19–19)] in mucinous cysts and 105 mg/dL (IQR: 96–127) in non-mucinous cysts ($p < 0.0001$).

For mucinous cyst diagnosis, CEA with the standard threshold (> 192 ng/mL) had a sensitivity and specificity of 72% (95% CI 51–88) and 96% (95% CI 82–100), respectively, and the ROC analysis revealed an area under the curve (AUC) of 0.842 (95% CI 0.726–0.959), as reported in Table 2a.

Glucose level in PCF, with a threshold < 50 mg/dL, had a sensitivity of 89% (95% CI 72–98), a specificity of 86% (95% CI 67–96), and an AUC of 0.86 (95% CI 0.748–0.973) for mucinous cyst diagnosis, as is reported in Table 2b.

A subgroup analysis of 12 resected cysts, with a surgically confirmed final diagnosis, showed an AUC of glucose that was marginally lower than the AUC of CEA, 0.972 (95% CI 0.727–1) and 1 (95% CI 1–1), respectively (Table 3).

Table 2 Glucose and CEA analysis in pancreatic cystic fluid (surgical and clinicopathologic diagnosis)

	Mucinous		Non-mucinous		*p* value
	Median	IQR	Median	IQR	
(a) Differentiation of mucinous ($n=28$) versus all other cysts ($n=28$)					
CEA (ng/mL)	741.0	165.0–28,566.5	9.0	5.0–18.5	< 0.0001
Glucose (mg/dL)	19.0	19.0–19.0	105.0	96.0–127.0	< 0.0001

	AUC	95% CI	*p* value	Cutoff	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
(b) ROC curve analysis of tumor markers in cyst fluids for diagnosis of mucinous pancreatic cysts ($n=28$ vs $n=28$)						
CEA	0.842	(0.726–0.959)	< 0.0001	> 192	72 (51–88)	96 (82–100)
Glucose	0.860	(0.748–0.973)	< 0.0001	< 50	89 (72–98)	86 (67–96)
CEA or glucose	0.853	(0.724–0.963)	< 0.0001	> 192, < 50	88 (70–98)	79 (59–92)

Table 3 Glucose and CEA analysis in pancreatic cystic fluid (surgical diagnosis)

	Mucinous		Non-mucinous		*p* value
	Median	IQR	Median	IQR	
(a) Differentiation of mucinous ($n=6$) versus all other cysts ($n=6$)					
CEA (ng/mL)	2600	808.5–186,672.0	11.5	6.88–92.0	0.01
Glucose (mg/dL)	19.0	14.3–36.8	96.0	87.0–127.8	0.006

	AUC	95% CI	*p* value	Cutoff	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
(b) ROC curve analysis of tumor markers in cyst fluids for diagnosis of mucinous pancreatic cysts ($n=6$ vs $n=6$)						
CEA	1	(1–1)	0.006	> 192	100 (48–100)	83 (36–100)
Glucose	0.972	(0.727–1)	0.022	< 50	83 (36–100)	100 (54–100)
CEA or glucose	1	(1–1)	0.004	> 192, < 50	100 (59–100)	80 (28–99)

A scatter plot dividing mucinous and non-mucinous categories into different cyst types included in each category, with glucose and CEA levels, is shown in Fig. 2a for all 56 cysts with a definitive diagnosis and in Fig. 2b for the subgroup of 12 cysts with a surgical pathology diagnosis.

The highest glucose reading obtained was 214 mg/dL in a non-mucinous cyst, a cystic NET, with all 17 SCAs with glucose levels above 95 mg/dL. Concerning lower glucose readings, there were 19 samples with glucose < 20 mg/dL, including 12 IPMNs, two MCNs, three ADCs, and two pseudocysts.

Additionally, we registered a reading error of the glucometer in 9/82 (10.9%) of PCF samples, all corresponding to mucinous cysts 9/38 (23.7%) as shown in Table 1. In these cases, high PCF viscosity precluded glucose reading using the glucometer.

The association of CEA and glucose levels did not significantly increase the differentiation between mucinous and non-mucinous cysts, with an increase in sensitivity but a reduction in specificity, as presented in Table 2.

Correlation of Glucose and CEA Assays

Table 4 shows the results of 19/82 patients, with glucose levels measured in fresh PCF, either with the glucometer at the time of EUS ($n=7$) or in the laboratory assay ($n=19$), which were compared to glucose levels measured a posteriori in the 82 frozen samples for this study. The results of glucose are highly reproducible using either technique, in fresh or frozen samples, except for marginally lower levels in laboratory evaluation, but without any change in cyst classification. In fact, there was a strong positive correlation between glucometer assay in frozen PCF and in-room fresh PCF ($r_S=0.860$ and $p<0.013$), as well as with laboratory glucose in fresh PCF ($r_S=0.874$ and $p<0.0001$).

Analyzing the association of CEA and glucose levels, there was a strong negative correlation between CEA and glucometer assay for glucose in frozen PCF ($r_S=-0.668$ and $p<0.0001$).

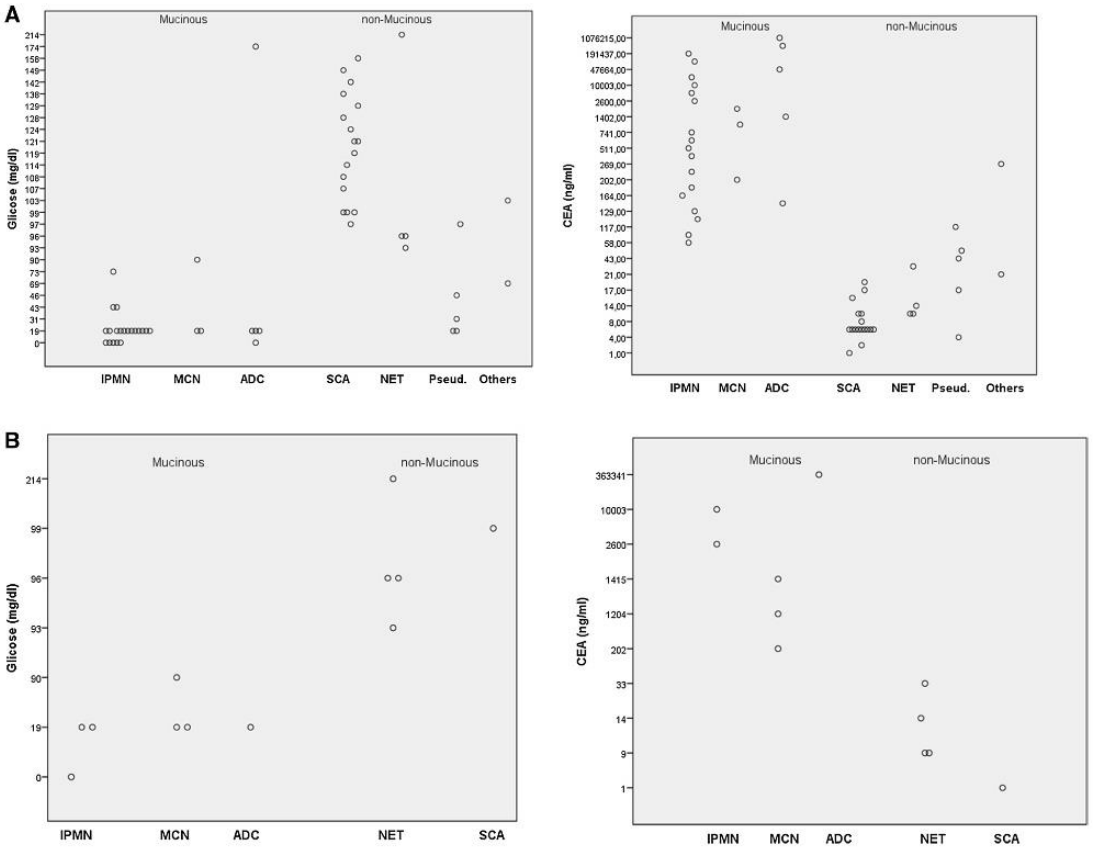

Fig. 2 Scatter plot displaying glucose (mg/dL) and CEA (ng/mL) levels in 56 mucinous and non-mucinous pancreatic cystic lesions including both surgical and clinicopathologic diagnoses (**a**) and only in 12 cystic lesions with a surgical pathology diagnosis (**b**). *IPMNs* intraductal papillary mucinous neoplasms, *MCN* mucinous cystic neoplasm, *ADC* adenocarcinoma, *SCA* serous cystadenoma, *NET* neuroendocrine tumor, *pseud* pseudocysts; others, lymphangioma and acinar cell carcinoma

Discussion

In this study, we tested the diagnostic accuracy of glucose levels on PCF samples obtained by EUS-FNA for pancreatic mucinous lesions and compared it to laboratory CEA level. We showed that by using a standard glucometer for cyst fluid analysis, a low glucose level (< 50 mg/dL) had the same predictive accuracy as an elevated CEA (> 192 ng/mL) for mucinous cyst diagnosis. Therefore, the excellent diagnostic performance with immediate on-site result, the low volume required, and the low cost make glucose assay using a glucometer an excellent biomarker for triage and diagnosis of mucinous cysts.

This study confirms two previous studies that reported high sensitivities, of 92% and 88%, respectively, of glucose measurement with a glucometer for mucinous cyst diagnosis, as compared to CEA measurement, which showed a substantially lower sensitivity, of 58% and 77%, respectively [14, 15]. This high sensitivity is clinically relevant, as a diagnostic test with higher sensitivity is more adequate to diagnose the largest number of lesions as possible. Using CEA level > 192 ng/mL as the criterion to diagnose mucinous cysts would exclude several lesions from surveillance, due to reduced sensitivity. In fact, in another previous study, the sensitivity of CEA was only 61%, with 39% of mucinous cysts misdiagnosed using CEA level alone [7]. This imperfect performance of CEA in mucinous cyst diagnosis may represent a lost opportunity for early diagnosis of pancreatic adenocarcinoma.

In our series, sensitivity of glucose and CEA for diagnosis of mucinous lesions was 89% and 72%, respectively, and specificity was 86% and 96%, respectively. Besides its

Table 4 Glucometer assay in PCF frozen samples, glucometer in EUS-FNA room, laboratory glucose, and CEA at time of EUS-FNA

Patients	Glucometer assay (frozen samples)[a]	Glucometer assay (EUS-room)[a]	Laboratory glucose[a]	CEA (ng/mL)
1	107	133	103	8
2	96		85	33
3	19		4	43
4	114		111	3
5	99		101	5
6	158	145	112	5
7	97		84	17
8	98	122	87	5
9	121		106	9
10	119	114	109	9
11	0	19	5	112
12	0		5	48
13	0		49	511
14	19		4	166
15	19		4	540
16	73		68	87
17	43		38	306
18	19	19	4	47,664
19	19	19	4	129

[a]Glucose (mg/dL); 0—reading error of glucometer; 4 (glucose < 5 mg/dL); 19 (glucose < 20 mg/dL)

higher sensitivity, the main advantages of glucose are its lower cost, on-site availability, and especially the fact that it requires such a small amount of fluid, < 2 µL. This compares to higher cost and logistical issues of laboratorial glucose and CEA assays, which require 50 µL and 200 µL of sample, respectively. Often, in clinical practice, low PCF volume precludes standard biochemical analysis, making the small amount of PCF required for glucometer analysis a major advantage. Furthermore, glucose measurement might be particularly useful in PCLs with CEA levels between 5 and 192 ng/mL and non-diagnostic cytology. In our series, this represented 34.1% (28/82) of samples that could have been erroneously classified as non-mucinous and excluded from the surveillance program.

It is also worth noting that we had nine reading errors of glucometer due to high viscosity, corresponding to almost a quarter of the mucinous samples, and occurring exclusively in mucinous cysts. This has not been reported previously but might become an increasing finding with more frequent use of this technique, occurring due to increased PCF viscosity in mucinous cysts. In our opinion, this should not be a problem, as the so-called string sign has been shown to have a positive predictive value of 94% for diagnosis of mucinous cysts in a study by Bick et al. [17].

As mentioned above, there are only two previously published studies showing that cyst fluid glucose has significant advantages over CEA and should be considered a routine diagnostic test for pancreatic mucinous cysts [14, 15]. However, in both studies, most patients were operated on, which might not reflect real-life practice, in which most PCLs do not require surgery. To our knowledge, this is the first study to include exclusively EUS-FNA obtained PCF samples, with most patients not being referred for surgery. However, a definitive cytological diagnosis was still available in 56/82 patients and all patients had a follow-up period greater than two years, which strongly supports the non-malignant nature of the lesions studied.

The AUC of glucose for mucinous cyst diagnosis was 0.86 in the 56 patients with a final surgical or clinicopathological diagnosis and 0.97 in the 12 patients with a surgical diagnosis, corroborating previous studies describing AUC of 0.88 [13], 0.89 [14], and 0.91 [15]. The lower AUC of glucose in clinical, compared to surgical series, may be due to a contamination of our clinical series by pseudocysts having a low glucose level similar to mucinous lesions and rarely require surgery. However, in clinical practice, pseudocysts are rare and, due to clinical context, rarely raise diagnostic issues with other PCLs. In an earlier study, low glucose level was also reported in the six pseudocysts included, with a median glucose level of 42 mg/dL, the lowest of benign PCLs [14]. In this particular clinical scenario, the EUS findings and CEA level can be complementary to glucose, allowing the diagnosis. On the other hand, glucose has some potential advantages over CEA as a biomarker. Its reproducibility is probably greater than CEA, as the latter optimal cutoff varies with different analyzers. In contrast, glucose levels measured either with glucometer or laboratory assay, in fresh or frozen samples, were highly reproducible, without any change in cyst classification from mucinous to non-mucinous. A limitation common to both biomarkers (glucose and CEA) is the ability to differentiate mucinous cysts but not malignant lesions requiring surgery.

Our study has several strengths. Its main strength is to analyze glucose level in PCF obtained preoperatively, by EUS-FNA, as is standard in clinical practice. While previous studies included mainly surgically treated patients, in our study, the diagnoses are predominantly clinicopathological, which certainly better represents daily clinical practice. Additionally, all data of patients were prospectively collected and registered, except for laboratory and "in-room" glucose levels that were retrieved from medical records, resulting in a significant proportion of patients, 95% (78/82), with simultaneous glucose and CEA evaluation. Identical to a previous study [14], the simultaneous evaluation did not significantly increase the identification of mucinous cysts.

The limitations of this study include the modest sample size and the restricted number of surgical pathology

diagnoses (12 out of 82 PCLs), with most diagnoses relying on clinicocytological features, possibly with diagnostic uncertainty. Nevertheless, this type of diagnosis with prolonged surveillance better reproduces clinical practice, in which non-surgical PCLs are predominant, while minimizing possible diagnostic imprecision. Our protocol of PCF storage minimizes pre-centrifugation processing delays and sample delay at room temperature, but even so, glucose is among the most labile metabolites [18] and the readings in frozen samples could have been affected, with consequent heterogeneity in data. However, this was hardly the case, as we found a strong positive correlation between glucometer assay in frozen PCF and in-room fresh PCF, as well as with laboratory glucose in fresh PCF. Finally, the limited number of PCLs excluded the evaluation of glucose in more pseudocysts and other rare PCLs, such as solid pseudopapillary neoplasm.

In conclusion, our study demonstrates that glucose level measured by a current glucometer is accurate for mucinous cyst diagnosis. Mucinous cysts present significantly lower glucose level than non-mucinous cysts, and almost a quarter of the mucinous samples displayed a reading error with the glucometer, due to increased viscosity, which itself also points to the diagnosis. Pseudocysts were found to be an exception, with low glucose levels, although being non-mucinous cystic lesions. These results suggest that in clinical practice, on-site measurement of glucose at time of EUS-FNA using a standard glucometer is a powerful tool that may complement or even replace CEA in mucinous cysts diagnosis, especially in small lesions with a limited amount of PCF. Nevertheless, before we accept these results as practice changing, larger prospective studies are needed.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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"It is not the end.
It is not even the beginning of the end…
It is the end of the beginning."

Winston Churchill