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The contribution of endoscopic ultrasound and biomarkers in the management of pancreatic adenocarcinoma and its precursor lesions

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THE CONTRIBUTION OF ENDOSCOPIC ULTRASOUND AND BIOMARKERS IN THE MANAGEMENT OF PANCREATIC ADENOCARCINOMA AND ITS PRECURSOR LESIONS

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ABBREVIATIONS

95%CI	95% confidence interval
ACG	American College of Gastroenterology
ACR	American College of Radiology
AGA	American Gastroenterological Association
AT	ataxia telangiectasia
AUC	area under the curve
AUROC	area under the receiver operating characteristics
BD-IPMN	branch duct intraductal papillary mucinous neoplasm
BPD	benign pancreatic disease
CA 19-9	carbohydrate antigen 19-9
CAPS	cancer of the pancreas screening consortium
CDKN2A	cyclin-dependent kinase inhibitor 2A
CEA	carcinoembryonic antigen
CEUS	contrast-enhanced endoscopic ultrasound
cfDNA	cell-free DNA
cfRNA	cell-free RNA
CP	chronic pancreatitis
crExos	circulating exosomes
CTC	circulating tumor cells
ctDNA	circulating tumor DNA
ctNAs	circulating tumor nucleic acids
ctRNA	circulating tumor RNA
DM	diabetes <i>mellitus</i>
DNA	deoxyribonucleic acid
DPC4	deleted in pancreatic cancer-4 (same as SMAD4)
DWI	diffusion-weighted imaging
ELISA	enzyme-linked immunosorbent assay
ERCP	endoscopic retrograde cholangio-pancreatography
ESGCTP	European Study Group on Cystic Tumours of the Pancreas
EUS	endoscopic ultrasound
EUS-FNA	endoscopic ultrasound-guided fine needle aspiration
EUS-FNB	endoscopic ultrasound-guided fine needle biopsy
EVs	extracellular vesicles
FACS	fluorescence-activated cell sorting
FAMMM	familial atypical multiple mole melanoma
FBG	fasting blood glucose
FDR	first-degree relative
FITC	fluorescein-5-isothiocyanate
FNA	fine needle aspiration
FNB	fine needle biopsy
FPC	familial pancreatic cancer
GPC I	glypican-I
GPC I + crExos	glypican-I positive circulating exosomes
GPI	glycosylphosphatidylinositol
HBOC	hereditary breast and ovarian cancer syndrome
HRIs	high-risk individuals
HRS	high-risk stigmata
HS	heparan sulfate
HSPG	heparan sulfate proteoglycans
ICG	International Consensus Guidelines (Fukuoka)
IPMN	intraductal papillary mucinous neoplasm
KRAS	Kirsten rat sarcoma viral oncogene
MCDT	multidetector computed tomography
MCL	mucinous cystic lesion
MCN	mucinous cystic neoplasm
MD-IPMN	main duct intraductal papillary mucinous neoplasm

MF	mass-forming
miRNA	microRNA
MMR	mismatch repair
MPD	main pancreatic duct
MRCP	magnetic resonance cholangio-pancreatography
MRI	magnetic resonance imaging
mRNA	messenger RNA
NFPTR	National Familial Pancreas Tumor Registry
NGS	next-generation sequencing
NLOD	new-late onset diabetes <i>mellitus</i>
NOD	new-onset diabetes <i>mellitus</i>
p16	protein 16
PA	pancreatic abnormalities
PanIN	pancreatic intraepithelial neoplasia
PanNET	pancreatic neuroendocrine tumor
PC	pancreatic cancer
PCPL	pancreatic cancer precursor lesions
PDAC	pancreatic ductal adenocarcinoma
PET	positron emission tomography
PJS	Peutz-Jeghers syndrome
PRSSI	serine protease 1 gene
RNA	ribonucleic acid
ROSE	rapid on-site evaluation
SCN	serous cystic neoplasm
SMAD4	mothers against decapentaplegic homolog 4 gene
TGF- β	transforming growth factor beta
TP53	tumor protein p53
TTNB	through-the-needle biopsy
US	abdominal ultrasonography
vs.	<i>versus</i>
WF	worrisome features
WHO	World Health Organization

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I. SUMMARY

Pancreatic ductal adenocarcinoma (PDAC) is a major problem worldwide, with increasing incidence and still disappointing high mortality rate. Its silent clinical progression, the lack of tools able to perform an early diagnosis along with the low efficacy of available treatments contribute to its dismal prognosis.

Meanwhile, between cancer initiation and its clinical manifestation there is a window of opportunity for early diagnosis of this disease. Therefore, efforts should be made to ameliorate the accuracy of imaging diagnosis and to look for biomarkers, easily accessible, able to detect cancer in early stage or, ideally, its high-risk precursor lesions. These lesions constitute the real targets suitable to prompt therapeutical intervention to improve patient's survival.

This PhD thesis was focused on the role of endoscopic ultrasound (EUS) combined with an upcoming and promising biomarker, glypican-I (GPCI), in the management of PDAC patients and its associated risk groups and precursor lesions.

In the **review articles** that constituted the background of this work, we discussed the facts related to PDAC burden and its associated risk conditions and the relevance of early diagnosis. One of the main focus should be to pursue strategies that allow the identification, among the at-risk population, the individuals harboring very high-risk conditions that should undergo screening and/or intensive surveillance programs. We demonstrated the importance of EUS as a premium technique in the management of patients with this neoplasia and its precursor lesions (mainly the mucinous cystic neoplasms), specifically its role in the diagnosis, allowing EUS-guided tissue acquisition for cytohistological confirmation, and local staging. Moreover, in the last decades, EUS has been gaining relevance also as a therapeutic tool for patients with PDAC. There is established evidence for its use in the management of PDAC-related symptoms, namely in obstructive jaundice resolution and control of pain, and a growing body of data supporting its feasibility, safety and efficacy (although mostly in experimental scenarios), as a mean to the delivery of anti-tumor therapies, such as injection of anti-tumor agents, assisting in radiotherapy, and guiding ablative modalities.

We have also reviewed and discussed the facts related to the present era of liquid biopsy, where research has focused on the search of biomarkers that can assist in the detection of neoplasias and its precursor lesions. In the field of PDAC, this search is more than urgent, as the only biomarker currently used in clinical practice, CA 19-9, lacks sensitivity and specificity, and is mainly used as a prognostic tool. We have described the several classes of molecules that have been pointed out for PDAC early diagnosis and management, and among them, we have highlighted the role of GPC I in circulating exosomes as a good candidate, since the findings reported by Melo et al. (1). In 2015 (1), data published showed this molecule presented absolute sensitivity and specificity in differentiating PDAC (independently of its stage) from non-PDAC controls, and offered considerable insights into the disease progression and tumor load.

Despite the heterogeneity of results among different studies that assess the role of GPC I as an early detection biomarker in PDAC, its potential deserves further investigation determining its value for PDAC diagnosis and prognosis. Moreover, as it seems to be overexpressed in some of PC precursor lesions, such as IPMNs, its potential contribution to the management of these patients deserves further insight.

Therefore, the **main aim** of this **investigational project** was to characterize, by EUS (with tissue acquisition whenever indicated), the pancreas of patients with PDAC or at increased risk for developing it, and to correlate imaging findings with the profile of the serum biomarkers GPC I and CA 19-9. Our **specific aims** were to:

1. Characterize the role EUS in the diagnosis and characterization of pancreatic mucinous cystic lesions;
2. Evaluate GPC I as potential tool in risk stratification for malignant transformation;
3. Evaluate the role of GPC I as a complement to EUS in discriminating PDAC from chronic pancreatitis (CP);
4. Access the combination of EUS and GPC I in PDAC screening among individuals with hereditary risk and patients with new-late onset diabetes *mellitus* (NLOD).

To answer our specific aims we have performed a cross-sectional prospective study, and gathered a cohort of patients with PDAC (n = 60), controls (n = 8), and patients belonging to one of the following associated risk groups: pancreatic mucinous cystic lesions (MCLs) (n = 40), CP (n = 29), NLOD (n = 20) and individuals with hereditary

risk for PDAC (kindreds of familial pancreatic cancer or individuals harboring an hereditary cancer syndrome with increased risk of PDAC) (n = 20).

This clinical study, after ethical committee approval, took place in the Department of Gastroenterology of *Centro Hospitalar Universitário de São João*, in Porto, Portugal, where all subjects were observed, examined, and followed-up. EUS was performed in all cases by three experienced endoscopists, using linear scopes from Olympus® and Pentax®, along with Olympus® EU-ME2 and Hitachi Avius® image processors. Doppler, elastographic and contrast-enhanced EUS (CEUS) (SonoVue®, Bracco, Italy) image acquisition was done in selected cases to assist the characterization of lesions. EUS-guided tissue acquisition [fine needle aspiration (FNA)/fine needle biopsy (FNB)] was performed whenever indicated, preferentially with a 22G FNA needle. A change of the needle caliber/type was considered if the macroscopic examination of the specimen seemed unsatisfactory or if dictated by rapid onsite evaluation (ROSE) information. All microscopic examinations were analyzed by experienced pathologists in pancreatic pathology and contextualized relative to the patients' clinical history and imaging data.

The molecular work concerning the distinct biomarkers was done at *Instituto de Investigação e Inovação em Saúde (i3S)*, University of Porto, Portugal. Briefly, at the moment of performance of EUS, a separate blood sample was collected and used for CA 19-9 analysis (ELISA) and GPC I quantification in circulating exosomes (ELISA and flow cytometry). Concentration as well as the mode size of circulating exosomes was also assessed.

Our study assembled comprehensive data on the combination of the use of EUS along with GPC I in the management of PDAC patients and its associated risk groups:

Our **results have demonstrated** that:

1. Patients with MCLs globally presented elevated levels of GPC I+ crExos, in the same magnitude of the levels registered in PDAC ones.
2. These levels were higher in cysts harboring “worrisome features” or “high risk stigmata” in the EUS evaluation.
3. GPC I+ crExos levels were also elevated among individuals with hereditary risk for PDAC (predominantly in those with history of previous cancer), even in absence of harmful pancreatic abnormalities in EUS examination.

4. NLOD patients presented low GPCI + crExos levels, similar to the control group.
5. Circulating exosomes positive for GPCI outperformed CA 19-9 as a biomarker to differentiate PDAC from CP. This can be particularly useful in the challenging mass-forming presentation of this inflammatory condition.
6. The performance of GPCI was equally good for both early and advanced stages of the tumor.

Despite some limitations of our investigational project (sample size, duration of follow-up, referential bias, technical restrains), we believe that longitudinal studies will clarify the potential of GPCI, along with EUS imaging, as a biomarker in PDAC management.

In summary, we have unraveled promising evidence for GPCI potential role in PDAC early detection and differential diagnosis, along with precursor lesions risk stratification, and in signaling individuals with genetic predisposition.

If this will be the case, and in complement with EUS exploration, GPCI acting both as a diagnostic and surveillance biomarker may substantially contribute to improve patient's management and survival.

2. RESUMO

O adenocarcinoma pancreático (ADCP) é um problema à escala mundial, com incidência crescente e uma ainda dececionante alta taxa de mortalidade. A sua evolução clínica silenciosa, a falta de ferramentas capazes de fazer um diagnóstico precoce e a baixa eficácia dos tratamentos disponíveis contribuem para este panorama sombrio.

No entanto, entre o início da carcinogénese e o aparecimento das manifestações clínicas existe uma grande janela de oportunidade para o diagnóstico precoce da doença. Portanto, devem ser feitos todos os esforços para melhorar a acuidade do diagnóstico por imagem e, sobretudo, na procura de biomarcadores, de fácil acesso, capazes de detetar o cancro em estágio inicial ou, idealmente, as suas lesões precursoras de alto risco. De facto, estas lesões constituem os alvos reais adequados para uma intervenção terapêutica atempada, de modo a melhorar a sobrevida dos doentes.

Esta tese de doutoramento focou-se no papel da ecoendoscopia combinada com um biomarcador investigacional promissor, o glipicano-I (GPCI), na abordagem clínica dos doentes com ADCP e suas lesões precursoras e grupos de risco associados.

Nos **artigos de revisão** que constituíram a base deste trabalho, discutimos os fatos relacionados com o flagelo que é o ADCP, as suas condições de risco associadas e a relevância do diagnóstico precoce. As estratégias de enriquecimento que permitam identificar, entre a população de risco, os indivíduos portadores de condições de muito alto risco candidatos a programas de rastreio e / ou vigilância intensiva devem constituir uma prioridade. Demonstrámos a importância da ecoendoscopia como uma técnica primordial na abordagem dos pacientes com esta neoplasia e suas lesões precursoras (principalmente as neoplasias císticas mucinosas), relevando o seu papel no diagnóstico, permitindo a aquisição ecoguiada de tecido para confirmação cito-histológica, bem como no estadiamento loco-regional. Além disso, importa sublinhar que a ecoendoscopia tem também ganho relevância como ferramenta terapêutica nos doentes com ADCP. Existem evidências estabelecidas para a sua utilização na resolução de sintomas relacionados com o tumor, nomeadamente no tratamento da icterícia obstrutiva e controlo da dor, e um número crescente de dados que apoiam a sua viabilidade, segurança e eficácia (embora principalmente em cenários experimentais), como um meio

para a administração de terapias diretas, como a injeção de agentes anti-tumorais, ou auxiliando a radioterapia e outras modalidades de ablação.

Revimos e discutimos também os fatos relacionados com a atual era da designada biópsia líquida, onde a investigação se tem centrado na procura de biomarcadores que possam auxiliar na deteção precoce de neoplasias e suas lesões precursoras. No campo do ADCP, esta investigação reveste-se de maior premência, visto que o único biomarcador atualmente utilizado na prática clínica, o CA 19-9, carece de sensibilidade e especificidade, sendo utilizado principalmente como ferramenta de prognóstico. Descrevemos as várias classes de moléculas que foram apontadas para o diagnóstico precoce do ADCP e, entre elas, destacamos o papel do GPCI nos exossomos circulantes. De forma importante, este biomarcador tem sido visto como um bom candidato, especialmente desde os resultados reportados por Melo et al. em 2015 (1), onde esta molécula apresentou sensibilidade e especificidade absolutas na diferenciação do ADCP (independentemente do seu estágio) de controlos não-ADCP, e ofereceu perspectivas consideráveis sobre a carga tumoral e progressão da doença.

Apesar da existência de alguma heterogeneidade nos resultados de diferentes estudos em relação ao papel do GPCI como biomarcador de deteção precoce, o seu potencial merece investigação adicional para determinar o seu valor no diagnóstico e prognóstico do ADCP. Além disso, como parece apresentar expressão aumentada em algumas lesões precursoras do ADCP - como é o caso das neoplasias mucinosas papilares intraductais - a sua potencial contribuição para individualizar a abordagem desses pacientes merece também a maior atenção.

Assim sendo, o **principal objetivo** do nosso **projeto de investigação** foi caracterizar, por ecoendoscopia (com aquisição de tecido sempre que indicado), o pâncreas de doentes com ADCP ou em risco aumentado para o desenvolver, e correlacionar os achados de imagem com o perfil de biomarcadores séricos, nomeadamente o GPCI e CA 19-9. Os **objetivos específicos** foram:

1. Caracterizar o papel da ecoendoscopia no diagnóstico e caracterização de lesões císticas mucinosas pancreáticas;
2. Avaliar o GPCI como ferramenta potencial na sua estratificação de risco para transformação maligna;

3. Avaliar o papel de GPC I como complemento da ecoendoscopia no diagnóstico diferencial entre o ADCP e a pancreatite crónica;
4. Avaliar a combinação da ecoendoscopia e GPC I no rastreio do ADCP nos indivíduos com risco hereditário e nos doentes com o diagnóstico recente (< 36 meses) de diabetes *mellitus* de início tardio (> 50 anos de idade).

Para dar resposta a estes objetivos, desenvolvemos um estudo transversal prospetivo, em que incluímos uma coorte de doentes com ADCP (n = 60), controlos (n = 8) e pertencentes a um dos seguintes grupos de risco: lesões císticas mucinosas pancreáticas (n = 40), pancreatite crónica (n = 29), diabetes *mellitus* de início tardio com diagnóstico recente (n = 20) e indivíduos com risco hereditário para ADCP (cancro pancreático familiar ou indivíduos portadores de síndrome de cancro hereditário com risco aumentado pra desenvolver ADCP) (n = 20).

Este estudo clínico, após aprovação da comissão de ética, decorreu no Serviço de Gastreenterologia do Centro Hospitalar Universitário de São João, no Porto, Portugal, onde todos os pacientes foram observados, examinados e acompanhados. A ecoendoscopia foi realizada em todos os casos por três endoscopistas experientes, usando ecoendoscópios lineares da Olympus® e Pentax®, juntamente com processadores de imagem Olympus® EU-ME2 e Hitachi Avius®. A aquisição de imagem Doppler, elastográfica e a utilização de ecoendoscopia com contraste ecográfico (SonoVue®, Bracco, Itália) foi realizada em casos selecionados para auxílio na caracterização das lesões. A aquisição de tecido guiada por ecoendoscopia (aspiração ou biópsia por agulha fina) foi realizada sempre que indicada, preferencialmente com agulha de aspiração com o calibre de 22G. Uma alteração do calibre / tipo da agulha foi considerada se o exame macroscópico da amostra pareceu insatisfatório ou se ditado por informação do patologista em casos de avaliação rápida no local. Todos os exames microscópicos foram analisados por patologistas experientes em patologia pancreática e contextualizados em relação à história clínica dos pacientes e aos dados dos exames de imagem realizados.

A análise molecular respeitante aos biomarcadores foi realizada no Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto, Portugal. Resumidamente, no momento da realização da ecoendoscopia, foi colhida uma amostra de sangue separada que foi usada para análise de CA 19-9 (ELISA) e quantificação do

GPCI em exossomas circulantes (cr Exos) (ELISA e citometria de fluxo). A concentração e a moda do tamanho dos exossomas foram também determinadas.

O estudo traduziu-se em dados elucidativos sobre a utilização conjunta da ecoendoscopia e do biomarcador GPCI na abordagem dos doentes com ADCP e pertencentes aos seus grupos de risco associados.

Os nossos **resultados demonstraram** que:

1. Os doentes com lesões císticas mucinosas apresentaram globalmente níveis elevados de GPCI+ crExos, na mesma magnitude dos registados nos casos de ADCP.
2. Estes níveis foram superiores nos cistos com “características preocupantes” ou “estigmas de alto risco” na avaliação ecoendoscópica.
3. Os níveis de GPCI+ crExos foram também elevados entre os indivíduos com risco hereditário para ADCP (predominantemente naqueles com história de prévia de cancro), mesmo na ausência de alterações pancreáticas na ecoendoscopia.
4. Os doentes com diabetes *mellitus* de início tardio com diagnóstico recente apresentaram níveis baixos de GPCI+ crExos, semelhantes aos verificados no grupo controlo.
5. Os exossomas circulantes positivos para GPCI superaram o CA 19-9 como um biomarcador capaz de discriminar o ADCP da pancreatite crónica, o que pode ser particularmente útil na apresentação pseudotumoral dessa condição inflamatória.
6. O desempenho do GPCI foi igualmente eficaz quer em situações de tumores em estádios iniciais quer avançados.

Apesar de algumas limitações do nosso projeto investigacional (tamanho da amostra, tempo de *follow-up*, viés de referência, restrições técnicas), cremos que estudos longitudinais irão permitir, no futuro, esclarecer a potencial utilidade do GPCI, em conjunto com a avaliação ecoendoscópica, na abordagem clínica do ADCP.

Em resumo, julgamos promissoras as evidências para o papel do GPCI na deteção precoce e diagnóstico diferencial do ADCP, assim como na estratificação de risco de

lesões precursoras e na sinalização de indivíduos com predisposição genética para esta neoplasia.

Se este for o caso, atuando a par da exploração ecoendoscópica como um biomarcador de diagnóstico e de vigilância, o GPC I poderá contribuir substancialmente para melhorar o manejo clínico e, de forma importante, a sobrevida dos doentes.

3. THESIS STRUCTURE

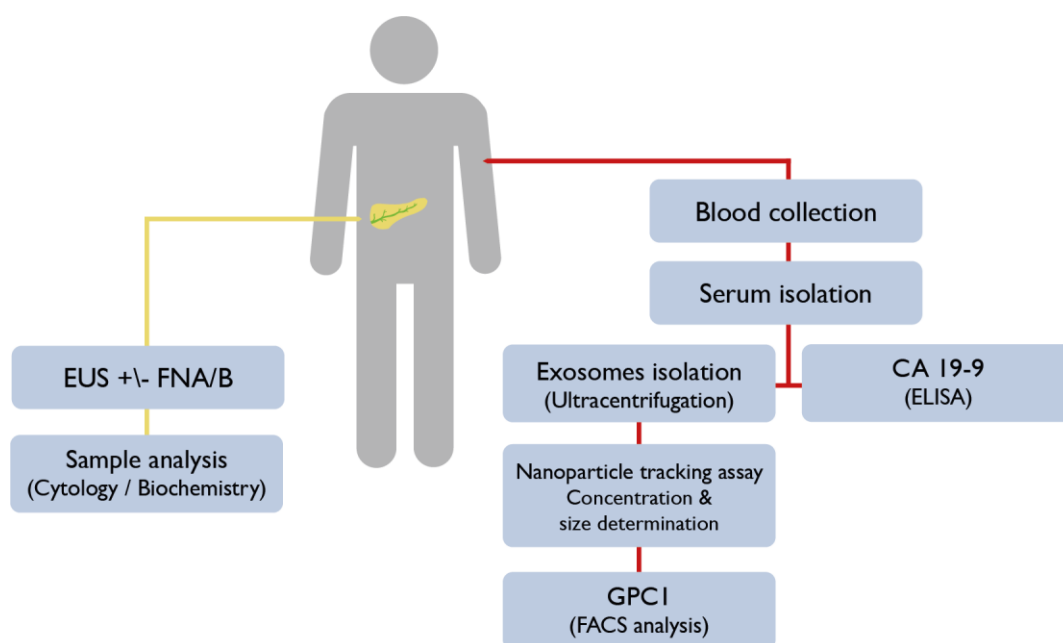
This PhD thesis addresses the theme of pancreatic adenocarcinoma focused on:

- PDAC and its precursor lesions;
- EUS as a tool in the management of these entities;
- Biomarkers, perceived as a promising hope to change the actual dismal outcome of this disease.

The main and specific **objectives** are listed in **chapter 3.1**.

Chapter 3.2. relates to **Introduction & Review of literature** and constitutes, along with the **Review articles** included in the **chapter 3.3.**, the background of this work. We have focused on the facts related to PDAC burden, its associated risk conditions, and in the importance of early diagnosis and treatment. We have highlighted the role of EUS as a premium technique in the management of patients with this neoplasia and its precursor lesions (mainly the mucinous cystic neoplasms). Finally, we have reviewed the evidence related to the current era of liquid biopsy and the relevance of the ongoing investigation on biomarkers that can assist in the diagnosis of PDAC in its early stages, in detection of high-risk precursor lesions, and as promising therapeutic targets.

Chapter 3.4. consists of the **Original research articles** that report the data gathered in this thesis. The main aim was to characterize by EUS (with tissue acquisition whenever indicated) the pancreas of PDAC patients and from individuals at increased risk, and to correlate imaging findings with serum biomarkers profile, namely GPC I and CA 19-9 (Figure A). This was a cross-sectional study of a prospective cohort of patients with PDAC, controls, and patients belonging to one of the following associated risk groups: pancreatic mucinous cystic lesions (MCLs), chronic pancreatitis (CP), new late-onset of diabetes *mellitus* (NLOD) and individuals with hereditary risk for PDAC (kindreds of familial pancreatic cancer or individuals harboring a hereditary cancer syndrome with increased risk of PDAC). This project had the ethical committee approval and took place at the Department of Gastroenterology of *Centro Hospitalar Universitário de São João*, Porto, Portugal, and at *Instituto de Investigação e Inovação em Saúde* (i3S), University of Porto, Porto, Portugal.

Figure A. *Investigational study flowchart.*

EUS: endoscopic ultrasound; FNA/B: fine needle aspiration/biopsy; ELISA: enzyme-linked immunosorbent assay; FACS: fluorescence activated cell sorting)

In **Discussion (chapter 3.5.)** we have highlighted the main findings and reflected about their potential implication in the management of PDAC and its associated conditions.

Chapter 3.6. points out the **Final remarks and Future directions** for upcoming research.

The **References** cited in this thesis are listed in **chapter 3.7.**

3.1. OBJECTIVES

The ultimate motivation for this thesis was driven by the hope of contributing to change the current dark scenario of PDAC.

The **main objective** of this thesis was to study the contribution of EUS and serum biomarkers in the management of PDAC and its precursor lesions.

The **specific aims** were:

1. Emphasize the importance of strategies allowing an early diagnosis of PDAC, a growing health burden in world population.
2. Highlight the need to identify and to access groups of individuals at increased PDAC risk.
3. Describe the role of EUS in PDAC management: from the diagnosis and staging to its contribution in patients' treatment.
4. Characterize the role of EUS in the diagnosis and characterization of pancreatic mucinous cysts and evaluate GPC I as potential tool in its risk stratification for malignant transformation.
5. Evaluate the role of EUS and GPC I in discriminating PDAC from CP.
6. Assess the combination of EUS with GPC I in PDAC screening among high-risk individuals.

3.2. INTRODUCTION & REVIEW OF LITERATURE

In this chapter, the literature background is presented in three sections, addressing the major topics of this thesis: pancreatic ductal adenocarcinoma (PDAC) and associated risk groups (3.2.1); the role of endoscopic ultrasound (EUS) in the management of PDAC and its precursor lesions (3.2.2); and, finally, the current era of biomarkers and liquid biopsy, with special emphasis on glypican-1 (GPC1) (3.2.3).

3.2.1. PDAC and associated risk groups

3.2.1.

1) PDAC: general considerations

The pancreas is a mixed gland, constituted by exocrine and endocrine lineages.

During embryonic development, the pancreatic cords, formed by undifferentiated pancreatic trunk epithelium, proliferate and differentiate into both tissues. The exocrine pancreas comprises acinar, ductal and centroacinar cells (2). Ductal cells are organized in a network of ducts that will drain the flow of digestive enzymes, which is secreted by acinar cells, the preponderant elements in the mature gland. Acinar cells, due to its plasticity, have the possibility to undergo metaplasia and give origin to ductal or ductal-like cells. This acinar-ductal metaplasia is a process that may be associated with neoplastic transformation (3).

The classification of pancreatic malignant neoplasms is currently based on the cellular differentiation of the neoplastic cells and on the macroscopic appearance (solid or cystic) of the tumor (4).

Pancreatic cancer (PC) is mainly divided into two types: those arising in the exocrine tissue, the carcinomas, which represent 95% of cases, and those arising in the endocrine tissue, the remaining 5%, the so-called pancreatic neuroendocrine tumors (PanNET) (5). PDAC constitutes the great majority of all pancreatic carcinomas, which also include rare forms, such as acinar cell carcinoma, acinar cell cystadenocarcinoma, among others (4, 6).

Being PDAC by far the most frequent type of PC (90% of all types), both designations are many times used indistinctly.

The main genetic background of PDAC carcinogenesis is formed by 4 frequently mutated genes (one oncogene and three tumor suppressor genes): KRAS (90%), CDKN2A/p16 (90%), TP53 (70%) and SMAD4 (55%) (2).

The translation of the mutated form of KRAS is an abnormal protein, constitutively activated, that fuels uncontrolled proliferation and survival pathways. The inactivation of the CDKN2A gene also leads to proliferative activity increment in result of the loss of protein 16 (p16), which major role is a negative regulation of the cell cycle, namely the G1 to S transition. In turn, inactivation of the tumor suppressor gene TP53, results in the cell escaping checkpoints that control DNA damage and apoptosis. Lastly, the frequent loss of SMAD4 gene results in the aberrant signaling by transforming growth factor beta (TGF- β), with important consequences at the level of PC microenvironment (2). Remarkably, this mutation on SMAD4 gene has been associated with poorer prognosis, with reported early recurrence and metastatic progression of the disease (7, 8).

The cause of PC is complex and multifactorial, being cigarette smoking and family history the most important recognized risk factors (9, 10).

PDAC is considered one of the deadliest human cancers. The defining features of PDAC biology that dictate the dismal natural history are the broad tumor heterogeneity, as well as the substantial crosstalk between the malignant epithelium and microenvironment components (11). In fact, this last aspect is pointed out as one of the most important factors in dictating the aggressive behavior of PC, mainly resultant from an intense desmoplastic reaction, with dense stroma formation (2). Pancreatic stellate cells play a central role in the genesis of this desmoplastic reaction, strictly associated with tumor formation, progression, invasion and metastasis. One of the explanations is based on the fact that the dense stroma restricts antitumor immunity, through its contribution to an immunosuppressive microenvironment. In addition, recent evidence demonstrated the ability of stellate cells to secrete valuable substances necessary to feed the tumor metabolism, such as alanine and glutamine-derived carbon, making it independent from glucose and other serum nutrients (12). In an innovative paper by

Ruivo et al. (13), also addressing the role of intratumor heterogeneity in cancer development, we have demonstrated that there is a cooperation network mediated by extracellular vesicles (EVs) led by cancer stem cells and agrin that allows the tumor to adapt and thrive. We postulate that therapy that targets agrin could have a direct impact in cancer stem cells, those ultimately responsible for feeding the tumor and resistance to treatment.

In recent years, PC has ranked the 14th most common cancer in the world, based on GLOBOCAN 2020, counting 495,773 new cases and being responsible for 466,003 deaths (4.7% of all deaths caused by cancer) in 2020 (14). Its incidence and mortality worldwide correlate with increasing age, and it is slightly more common in men than in women (14).

In the past few decades, despite progression in the knowledge of potential risk factors and newly available tools for early diagnosis, the incidence of PC has continuously increased. Moreover, its prognosis remained poor, with a 5-year survival rate < 10% for all stages analyzed together, and of 20% and 2% for patients with localized and distant metastatic diseases, respectively (15).

At present, it is the 3rd leading cause of cancer-related deaths in the United States, and is expected to become the 2nd within the next decade (16, 17). These dismal figures are attributed mainly to a late and incurable stage at diagnosis, along with a characteristic tumor resistance to conventional therapies. In fact, a late-stage diagnosis makes most treatment options ineffective (18).

Nevertheless, a slight increase in the overall 5-years survival rate from 6% to 9%, has been observed from 2014 to 2018, which shows that progress is being made (15). These small survival improvements have been attributed mainly to the increased use of sectional imaging techniques, diagnosing at less advanced stages, along with a decrease in surgical morbidity and mortality rates, and developments in neoadjuvant and adjuvant therapies (19).

Some breakthroughs have also been made in the past few years in the treatment of a small population of patients with inherited deficiencies in DNA damage repair

At its early stages, PC usually is clinically silent, but upon progression of the tumor, a gradual onset of non-specific symptoms including jaundice, weight loss, pale stools, abdominal pain and fatigue are usually seen (20).

The overwhelming majority of patients with PDAC continues to present with locally advanced or distant metastatic disease (80-85%), and only a minority of patients are candidates for surgical resection (15-20%), the only potentially curative treatment that increases survival (4, 21).

In limited clinical series from the past, it was documented that patients with incidentally discovered PDAC, especially those with sub-centimeter lesions, had better survival rates (22, 23). More recently, data from patients on longitudinal surveillance for PDAC incidence due to familial risk also supports the idea that earlier diagnosis is associated with increased survival, albeit not always “cure”(24, 25). This makes imperative to assure all the efforts to detect pancreatic tumors in its initial stages. Moreover, as treatment options for resectable diseases continue to improve, including neoadjuvant multimodality therapy, together with more aggressive adjuvant regimens (26, 27), a “stage shift” from the current 15-20% resectable proportion to 50% or greater would definitely lead to improve the prognosis of this dismal disease (28).

In PDAC, equally to the vast majority of other cancers, treatment options depend essentially on the stage of the disease at diagnosis.

Based on the clinical stage of the tumor, PC is classified into four types:

- Stage I (no spread or resectable), the tumor is limited to the pancreas and has 2 cm or less (IA) or is greater than 2 cm but less than 4 cm (IB);
- Stage II (local spread or borderline resectable), the tumor is > 4 cm and is limited to the pancreas, or there is local spread to the nearby lymph nodes;
- Stage III (wider spread or unresectable), tumor may have expanded to the nearby blood vessels or nerves, but has not metastasized to distant sites;
- Stage IV (metastatic), cancer has spread to distant organs (29).

Patients with promptly resectable disease are usually proposed to upfront surgery. However, this practice can be changed in a near future, as important evidence is being collected by ongoing trials (NEONAX, Prep-02/JSAP05 and PREOPANC-1) (30-32),

suggesting that neoadjuvant chemo or chemoradiotherapy may have clinical advantages over upfront surgery in early-stage PC.

In locally advanced and borderline resectable tumors, the downstaging has been shown to be achieved in 30-90% of cases, allowing resection with curative intent (33, 34).

Regarding adjuvant treatment after resection of the tumor, a multicenter, open-label, phase 3 randomized clinical trial (ESPAC-4 trial) showed that the combination of gemcitabine and capecitabine was superior to gemcitabine on monotherapy, albeit with modest increase in survival (35). A more recent trial (PRODIGE 24/CCTG) revealed that adjuvant therapy with a modified FOLFIRINOX regimen led to significantly longer survival than gemcitabine among patients with resected PC (median overall survival of 54.4 months in the modified-FOLFIRINOX group *versus* (vs.) 35.0 months in the gemcitabine group; $p = 0.003$), although a higher incidence of toxic effects was described (36).

Advanced stage tumors have been typically treated with gemcitabine, with modest increase in median survival from 3 to 6 months when compared to untreated patients (37). Several controlled randomized trials evaluating outcomes with novel chemotherapy agents and combinations with good biologic base (such as FOLFIRINOX) have been recently published, but median survival has not increased substantially beyond 6 months (38).

Interestingly, it appears that patients harboring BRCA1/2 germline mutations have better chance to be responsive to chemotherapy. In fact, increased rate of complete pathologic response after neoadjuvant FOLFIRINOX was registered among BRCA mutation carriers with borderline resectable PC (39). Also, a randomized, placebo-controlled trial showed improved progression-free survival in patients with metastatic PC under maintenance treatment with a poly (adenosine diphosphate-ribose) polymerase inhibitor (Olaparib) when germline BRCA mutations were present (40).

Nevertheless, although a remarkable effort and commitment to perform clinical trials, little has been gained in overall survival of patients with advanced PDAC so far (41, 42).

Identifying PDAC as early as possible is essential, but there are still several challenges. On one hand, despite its high mortality, PDAC is epidemiologically considered relatively uncommon, with an overall 1.3%-1.5% lifetime risk of developing the disease (14). This low prevalence makes screening adult population unfeasible. On the other hand, the existing methods of diagnosis would incur in unacceptably high rates of false-positive findings, which could lead to consequent substantial implications (43).

Therefore, the consensus in most countries is that widespread population-based screening for PDAC, or even in patients with only one affected first-degree relative (FDR), is neither practicable nor indicated (44).

However, a different scenario is pursuing early detection in patients with higher risk for PDAC, most of them with hereditary conditions predisposing the development of this neoplasia. Several other groups at high risk have been identified, including individuals with MCLs of the pancreas, people older than 50 years newly diagnosed with type 2 diabetes *mellitus* (DM) and individuals with CP (28, 44).

Clinical risk factors are relatively broad and non-specific. Despite extensive research, there are only few universally recognized risk factors for PC such as: age, exposure to tobacco smoking, heavy alcohol intake, obesity and DM (45).

Meanwhile, in a meta-analysis, different occupational exposures such as pesticides, hydrocarbons, heavy metals, suspended particles, ionizing radiation or nitrosamines were associated with increased risk for PDAC (46). Some other environmental factors, such as consumption red meat, sedentary lifestyle, and infectious agents like *helicobacter pylori*, hepatitis B and C virus have been inconsistently referred as potential risk factors (47).

Undoubtably, cigarette smoking is by far the main recognized risk factor for PDAC, contributing for a third of all cases in most areas of the world. Its carcinogens and metabolites may have both genetic and epigenetic effects at early and later stages in the tumorigenesis (48). In terms of time of exposure, being a current smoker (compared with never smoking) significantly advances the risk of developing PC by 7.6 years (49). Having quit for more than five years appears to reduce the risk to the one of nonsmokers (48).

Although conflicting, some experimental data suggested a possible inhibitory effect of aspirin and other nonsteroidal anti-inflammatory drugs on pancreatic tumorigenesis (50).

3.2.1.

II) PDAC associated risk groups

The following topics refer to the subpopulations of individuals harboring established conditions that increase the risk for PDAC development in their lifetime.

A) Familial or inherited risk

Most PDAC are sporadic, but up to 10% may be due to an underlying genetic predisposition. These include familial pancreatic cancer (FPC) in 7%, and the individuals harboring other hereditary cancer syndromes in 3% (44).

This heterogeneous group of subjects presents a theoretical lifetime cumulative risk of PC over 5% (51).

In Table I, it is summarized the risk of PC in patients harboring syndromes associated with high-risk germline mutations (52).

Table I. Risk for PC in patients with high-risk germline mutations

Gene(s)	Common name	Risk of PC
STK11/LKB1	Peutz-Jeghers syndrome	RR 132 (95% CI, 44-261)
PRSS1	Hereditary pancreatitis	SIR 53 (95% CI, 23-105)
CDKN2A	Familial atypical multiple mole/melanoma syndrome	RR 13-39
MLH1, MSH2, MSH6	Lynch syndrome	RR 8.6-11
TP53	Li-Fraumeni syndrome	RR 7.3 (95% CI, 2-19)
ATM	Ataxia-telangiectasia	RR 3.92 (95% CI, 0.44-14.2)
BRCA1	Hereditary breast and ovarian cancer	RR 2.26 (95% CI, 1.26-4.06)
BRCA2, PALB2	Hereditary breast and ovarian cancer	RR 3.5-6.2 (95% CI, 1.87-6.58)
Familial PC in 1 or 2 first-degree relatives (several genes)	Familial pancreatic cancer	RR 4-9.3

RR, relative risk; SIR, standardized incidence ratio

Adapted from DaVee et al. (52).

Familial pancreatic cancer (FPC) is defined as a family with at least one pair of first-degree relatives (parent-child or sibling pair) with PC without an identifiable predisposing cancer syndrome in the family (53).

In 1967, Lynch et al. (54) first recognized family history as a risk factor for PC, when this cancer was reported in an adenocarcinoma-prone family.

After that, several case-control and prospective cohort studies have demonstrated an increased risk of PC among subjects with family history of PC (55, 56). In a prospective follow-up held by the National Familial Pancreas Tumor Registry (NFPTR), involving more than 5000 individuals from 838 kindreds (370 FPC kindreds and 468 sporadic PC kindreds), Klein et al. (10) estimated the relative risk of the development of PC in FPC kindreds to be 6.4 (95% CI: 1.8-16.4) and 32.0 (95% CI, 10.2-74.7) for individuals with 2 or 3 affected FDR, respectively.

While pathogenic germline mutations in specific genes have been associated with an increased risk of PC (from 4% to 40%), a causal germline mutation is identified in fewer than 20% of these families (57, 58). Meanwhile, recent recommendations advise that all individuals with newly diagnosed PDAC undergo germline genetic testing to potentially guide treatment options (59). With this strategy, ultimately, mutation carriers could be more readily identified, and their family members would benefit from testing for the familial gene.

Several **hereditary cancer syndromes** and corresponding gene abnormalities have been described to be associated with increased risk for developing PDAC. At present, there are known inherited gene mutations as seen in hereditary pancreatitis and in inherited cancer syndromes. The most significant ones are: Peutz-Jeghers syndrome (PJS), Lynch syndrome, hereditary breast and ovarian cancer syndrome (HBOC), familial atypical multiple mole melanoma (FAMMM), and ataxia telangiectasia (AT) (60).

PJS is an autosomal dominant, hamartomatous polyposis syndrome, with high penetrance. It is caused by inherited mutations in the *STK11/LKB1* gene, with an estimated relative risk of PDAC of 132 (CI 95% 44-261) (61).

Lynch syndrome is an autosomal dominant inherited disorder, caused by germline mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2*. Individuals with Lynch syndrome have an estimated lifetime risk of developing PDAC of 3.7% (8.6-fold higher than general population risk) (62).

HBOC syndrome is associated with germline mutations in two cancer susceptibility genes: *BRCA1* and *BRCA2*. These gene mutation carriers have a significantly increased

lifetime risk for developing breast and ovarian cancer, as high as 84% and 39% respectively (63). BRCA2 mutations are found in as many as 12-17% of patients with FPC and can be present even in the absence of breast or ovarian cancer, as well as in apparently sporadic PC (63). Partner and localizer of BRCA2 (PALB2) is another associated inheritable gene recently described as a PDAC susceptibility gene, which mutations may be causative for 3-4% of FPC (64). BRCA1 mutation-positive families have a risk for PC 2- to 3-fold greater than general population. In turn, germline BRCA2 mutations have been associated with a relative risk of developing PDAC of 3.51 (CI, 1.87-6.58) (60).

FAMMM is another autosomal dominantly inherited syndrome with incomplete penetrance. Germline mutations in the p16/CDKN2A gene are associated with FAMMM, although there has been wide variability in its the reported prevalence. The risk of PDAC in kindreds with FAMMM is 13- to 39-fold greater than the average population (52).

Hereditary pancreatitis, although representing a very small fraction of PDAC cases, is associated with a markedly increased risk of this neoplasia. It is an autosomal dominant disorder, most often associated with mutations in serine protease 1 gene (PRSS1). Affected family members have a cumulative risk of PDAC of 10, 19, and 54% calculated at 50, 60, and 75 years, respectively (65).

Ataxia telangiectasia (AT) is a rare autosomal recessive condition characterized by early onset progressive cerebellar ataxia, telangiectasia of the skin, ionizing radiation sensitivity and immunodeficiency. This disorder presents during the first decade of life in biallelic mutation carriers and these individuals carry a 38% risk for cancer. The gene affected is the ATM gene. Monoallelic mutation carriers also harbor cancer risks, including breast and pancreas (66).

In all these high-risk individuals (HRI), kindreds of FPC and hereditary cancer syndromes, screening is mainly based on annual magnetic resonance imaging (MRI) and EUS. The goal is to identify early-stage cancers or high-risk precancerous lesions [pancreatic intraepithelial neoplasia (PanIN) or intraductal papillary mucinous neoplasms (IPMN) with high-grade dysplasia], amenable to surgical treatment (58, 67).

B) Mucinous cystic lesions

Pancreatic cysts are found in nearly 8% of individuals older than 70 years (68). Their importance is related to the fact that 2 of the 3 recognized precursor lesions of PDAC are, in fact, cystic lesions: the IPMN and the mucinous cystic neoplasms (MCN) (18).

Up to 15% of PDAC are thought to arise from IPMNs and MCNs, collectively referred to as MCLs (28).

In contrast to the third precursor lesion, PanIN, which can only be identified on histopathology, pancreatic cysts are easy to detect and therefore can be a target for potential early detection of PDAC (69).

Nevertheless, two issues muddle this simple concept. First, many pancreatic cysts are not mucinous neoplasms and constitute lesions with no risk of malignant transformation and thus do not require surveillance. Second, most of the IPMN and MCN do not progress to PDAC (18).

Among the mucinous cysts, most of them are IPMNs, which can be generally categorized based on location and extent of involvement within the gland, as main-duct (MD-IPMN), branch-duct (BD-IPMN) and mixed type (MT-IPMN) (70). MD-IPMNs account for 15-21% of IPMNs, are often located within the pancreatic head and are characterized by a segmental or diffuse dilatation (> 5 mm in diameter) of the main pancreatic duct (MPD), for which other causes of ductal obstruction have been excluded (71). BD-IPMNs account for 41-64% of IPMNs, are distributed throughout the pancreas and are frequently multifocal (71).

IPMNs have been also classified into four histopathological types based on the cytoarchitectural features and immunophenotypes: gastric (49-63%), intestinal (18-36%), pancreaticobiliary (7-18%), and oncocytic (1-8%) types (72, 73). The latter has been recently removed from the IPMN group, to be classified as intraductal oncocytic papillary neoplasm (IOPN) by the new WHO classification of Digestive Tumours (74). While the intestinal type is the most frequent among MD-IPMN and carries the higher risk of invasive carcinoma, the gastric type is the most common subtype among BD-IPMN and it is usually characterized by a low proliferative activity with a very low risk of malignant transformation (75, 76).

MCNs are defined as a rare mucin-producing cyst forming epithelial neoplasia with a distinctive ovarian-type stroma, typically developing in female individuals. Usually, they are solitary, predominantly located in the body and tail of the pancreas, and do not communicate with the ductal system (77).

The clinical tools currently available are imperfect at differentiating between benign cysts that are safe to discharge, MCLs that have low-grade dysplasia and are safe to watch, and MCLs that harbor high-grade dysplasia or invasive cancer and require surgical resection.

This limitation is a considerable problem, as highlighted by the fact that 25% of patients who have a presumed mucinous cystic lesion removed are ultimately found to have a cyst with no malignant potential (78). This assumes critical importance when considering the operative mortality (4-10%) and morbidity (40-50%) of these procedures (79, 80).

In the absence of a perfect test, the assessment and management of pancreatic cysts demands a multidisciplinary approach, considering the clinical presentation, MRI/EUS imaging and analysis of cystic fluid components.

In recent series, either from the occidental and oriental parts of the world (81, 82), the long-term risk of pancreatic malignancy in patients with BD-IPMN has been assessed in large cohorts. Pergolini et al. (81) described the development of high-grade dysplasia or invasive neoplasm in 5.5% of cases after a 5-year period of follow-up, while Oyama et al. (82), in over 1400 consecutive patients under surveillance, reported overall incidence rates of PC (including cases resulting from cyst degeneration and concomitant cancers) of 3.3%, 6.6%, and 15.0% at 5, 10, and 15 years, respectively.

Even with drawbacks of EUS, such as being an operator-dependent procedure and relatively invasive nature in comparison with cross-sectional studies, EUS, and especially EUS-guided FNA, has been widely used to investigate and stratify pancreatic cystic lesions (Figure B) (83).

Figure B. EUS in the characterization of pancreatic cysts.



- A. Simple bilobulated cyst in the pancreatic body.
 B. BD-IPM with septa, areas of focal wall focal thickening and a mural nodule (white arrow).
 C. EUS-FNB targeting a mural nodule in a MD-IPMN.

Adapted from Moutinho-Ribeiro P. et al. World J Gastroenterol. 2022. (84)

A meta-analysis including 41 studies evaluating cyst features and the risk of malignancy in IPMN revealed that: a cyst size > 3 cm, presence of mural nodules, dilatation of the main pancreatic duct, and main vs. branch duct IPMN were independent risk factors associated with malignant transformation (85). The presence and the size of mural nodule has been consistently pointed out as one of the most important red flags for prediction of high-grade dysplasia or invasive carcinoma (86).

Cyst fluid samples collected by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) have been extensively studied to discriminate mucinous from non-mucinous cysts, and to access malignant transformations in the formers. A meta-analysis including 18 studies, showed a pooled sensitivity of 54% and specificity of 93% of cytological diagnostic capability for differentiating mucinous from non-mucinous cysts (87).

Given the fact that aspirated cyst fluid seldom contains enough cellular elements to allow a proper cytological diagnosis, investigation has been focused on the role of tumor markers and amylase content.

In current clinical practice, carcinoembryonic antigen (CEA) is the most frequently used tumor marker for distinguishing mucinous from non-mucinous cysts, with an optimal calculated cut-off of 192 ng/mL (88). Meanwhile, cyst fluid CEA levels are not accurate for distinguishing malignant from benign cystic lesions (89).

In relation to amylase level, cysts with concentration > 250 U/L are likely to be IPMN or pseudocysts, whereas levels < 250 U/L are typically seen in MCN or serous cystic neoplasm (SCN) (90). More recently, intra-cyst low glucose levels (≤ 50 mg/dl) were

shown to be an accurate additional parameter in the differentiation of mucinous from non-mucinous pancreatic cysts (91-93).

Within the past decade, research has been conducted for the identification of novel biomarkers, including DNA, microRNA (miRNA), protein and metabolite-based molecules, in order to accurately classify pancreatic cysts and assess its risk of malignancy (94).

In this field, increasing interest has been documented in molecular analysis through next-generation sequencing (NGS), a very sensitive technique for detection of genetic alterations in predefined cancer gene panels, even in samples with limited DNA content, such as pancreatic cyst fluid (95, 96).

In a recent systematic review and meta-analysis, molecular analysis for KRAS + GNAS mutations in EUS-acquired pancreatic cyst fluid revealed high sensitivity and specificity with significantly improved diagnostic accuracy for diagnosis of IPMNs and MCNs when compared with CEA alone (97).

In turn, two recent advances have occurred in endoscopy. First, with the development of confocal endomicroscopes that can be passed, through a 19-gauge needle, into the cyst cavity, providing in-vivo real-time imaging of the cyst epithelium at microscopic level (98, 99). Second, with the possibility of performing EUS-guided through-the-needle biopsy sampling of the lining of pancreatic cystic lesions with a dedicated microforceps (100-103).

Unfortunately, at present days, there is still inability to accurately predict malignant transformation, for instance of a BD-IPMN, the most frequent mucinous pancreatic cyst. So, within any patient's lifetime, an appropriate surveillance is required, tailored by individual risk factors, clinical, imaging and cystic fluid findings.

A myriad of consensus and evidence-based guidelines for pancreatic cysts management, and, specifically, for BD-IPMNs, have been developed by several medical societies, namely the International Association of Pancreatology (Revisions of International Consensus - Fukuoka), the American Gastroenterological Association (AGA), American College of Gastroenterology (ACG), the American College of Radiology (ACR) and the European Study Group on Cystic Tumours of the Pancreas (ESGCTP) (104-108). While the surveillance strategy differs between these guidelines,

they are all in agreement that the risk of malignancy should be weighed against comorbidities and life expectancy of a given patient.

Ideally, a novel approach to the management of pancreatic cysts that combines the most specific clinical features with upcoming molecular markers would be more than welcome, with the aim to differentiate the small number of individuals with MCLs who have the highest risk of progression to PDAC (109-113).

C) New-onset diabetes *mellitus*

The association between diabetes DM and PDAC has been known since the 1800s (114), however, the complex and multidirectional relationship between the two entities is yet to be fully understood.

PDAC is known to be diabetogenic, residing in one of the strongest and most consistent diabetogenic forces known to humans. It impairs glucose homeostasis in nearly all subjects in whom it occurs (115).

It has been documented that late onset (> 50 years of age) DM is associated with a 6- to 8- fold increased risk of developing PDAC in 3 years. Nevertheless, only approximately 1% of late onset DM subjects will develop PDAC (116).

While long-standing type 2 DM is a modest risk factor for PDAC (1.5 to 2-fold increased risk), new-onset DM (NOD) is now considered to be a manifestation and harbinger of the disease (115). Increasing epidemiological, experimental and clinical evidence supports that NOD can be a clinical manifestation of asymptomatic PDAC and offers the promise for early detection of PDAC in these individuals (28).

Nearly 0.8–1% of individuals aged over 50 years with NOD have DM secondary to PDAC. However, distinguishing PDAC-associated DM from the more prevalent type 2 DM is challenging, and there is an urgent need to improved strategies to better identify those with PDAC in the NOD group (18).

It is estimated that in individuals who have had type 2 DM for less than 1 year, the relative risk of PDAC increases to 5.4-fold (117), with considerable investigation indicating that the hyperglycemia state and diabetes can be induced by the tumor (118).

These findings were supported in a population-based cohort of PDAC patients, from Olmsted County, Minnesota, United States, in whom fasting blood glucose (FBG) levels were used to describe the glycemic status of all PDAC patients. In this study, it was reported that 42% met the American Diabetes Association criteria for DM (of which 52% were NOD), 13% had advanced pre-DM (defined as FBG > 120 mg/dl), 21% have impaired FBG and only 9% had a normal FBG level at PDAC diagnosis. The observation that nearly 85% and 50% of patients had either elevated FBG or DM, respectively, at time of PDAC diagnosis, underlines that disturbance of glucose homeostasis is an almost universal phenomenon in this type of cancer (119).

Importantly, in another recent study from Sharma et al. (120), FBG levels were plotted in PDAC and matched controls up to 60 months prior to PDAC diagnosis. From 60 to 36 months, these levels were similar in cases and controls. Starting 30-36 months before PDAC diagnosis, glucose levels gradually rose, crossing the DM threshold (126 mg/dl) around 6-12 months before diagnosis. Using clinical-based resected PDAC subjects, the same study, also demonstrated that FBG levels correlated with tumor volume, with FBG levels starting to rise when tumors reached 1-2 cc and overpassing the DM threshold around 12 cc in volume (120).

The observation that the rise in blood glucose in PDAC occurs well before visible appearance of tumor, suggests that DM in PDAC cannot be attributed purely to destruction of the gland (119, 120). Some emerging data explored the concept of PDAC-induced paraneoplastic DM, including the identification of potential mediators of beta-cell dysfunction, such as adrenomedullin (121). In agreement with this, is the fact that PDAC has been shown to cause insulin resistance and beta cell dysfunction, which resolve with tumor resection (119).

Promisingly, a model (END-PAC) to determine the risk of PC within 3 years after the diagnosis of NOD, in patients older than 50 years, based on change in weight, change in blood glucose, and age at onset of DM was recently reported (122). Meanwhile, it needs further validation with an independent prospective study.

Lastly, an ongoing project initiated by the Consortium for the Study of Chronic Pancreatitis, Diabetes, and Pancreatic Cancer (CPDPC), with an estimated cohort of

10,000 subjects 50 years or older with NOD, aims to estimate the 3-year probability of PDAC development, to establish a biobank of clinically annotated biospecimens from pre-symptomatic cancer and control subjects, and also to conduct phase 3 validation studies of promising biomarkers for identification of incident PDAC. Ultimately the results would provide a platform for development of a future screening protocol for early PDAC detection among NOD patients (123).

In summary, all these findings strongly suggest hyperglycemia is as biomarker of early invasive PDAC, and the diabetes experienced by most patients with PDAC is of recent onset (diagnosed less than 24-36 months before diagnosis of the tumor). This offers an excellent opportunity for early detection of PDAC among this patients' group of risk.

D) Chronic pancreatitis

Chronic pancreatitis is a debilitating disease of diverse etiology and equally varied manifestations. In addition to causing severe pain, diabetes and exocrine insufficiency, CP increases the risk for developing PDAC (124).

Long-standing CP has been considered as a strong risk factor for PDAC, with a lag period between both diagnosis, in general, of 1 or 2 decades (125). Nevertheless, epidemiological observations have shown that although there is a strong link between CP and PDAC, only 5% of patients with this condition will develop PDAC over a 20-year period (126).

Caution should be taken when analyzing the relationship between these two entities, especially if the diagnosis of PDAC is made within a 2-year period after the diagnosis of CP. Having in mind that only a small fraction of PDAC patients (~5%) present with acute pancreatitis at the time of cancer diagnosis (127), pancreatitis appearing a year or 2 before the diagnosis of PDAC is often the result of tumor-related ductal obstruction and, therefore, can be a clinical manifestation of PDAC. Studies have showed that the likelihood of a diagnosis of PDAC after an episode of acute pancreatitis is ~1%, being highest in the first 2 years (127).

The short-term strong association between CP and PDAC risk can also be due to initial misclassification of a PC as CP. This is especially true when considering the high

demanding scenario of mass-forming presentation of the inflammatory process. The risk of such misclassification is high due to similarities in clinical, radiological, and biochemical nature of these two diseases (128).

It is believed that a causal relationship may exist between CP and PC. As inflammation accumulates in the pancreas, stellate cell activation may promote carcinogenesis in genetically predisposed individuals (129). Supporting that, is the fact that mutations in the KRAS oncogene, that are seen in about 90% of all PCs, have also been found in patients with CP, indicating a possible common pathway for both diseases (130).

Moreover, it has to be stressed that both conditions share well-established risk factors, namely exposure to tobacco smoking and alcohol consumption (131).

A recent systematic review and meta-analysis was conducted to examine the association between CP and PDAC with special emphasis on the temporality of this association (132).

When the cases of cancers occurring in the first 2 years following a diagnosis of CP were excluded, the risk of PDAC was elevated 16-fold. While this risk declines over time, it persists high even after a long period of follow-up. The risk was still 8-fold increased when considering a 5-year lag period from CP to PDAC diagnosis and persisted elevated for almost 10 years (132).

Since only a small proportion of CP patients will develop sporadic PDAC, at the present time, using them as a potential high-risk group for screening or early detection should take in consideration enrichment strategies to identify in advance the eligible subset of individuals at very high-risk.

3.2.1.

III) Early detection of PDAC: a window of opportunity

There is an urgent need for prompt PDAC diagnosis, and a shift toward its early detection has been recommended by several health-care institutions (18).

The benefits of PDAC early detection is supported by numerous observations. Patients diagnosed with stage I disease experience longer survival (133). Moreover, PDAC diagnosed by incident (i.e., discovered by chance while investigation of other diseases) is also associated with longer median survival when compared to those cases diagnosed in symptomatic patients with complaints attributed to the tumor (133).

The scenario of PDAC patients detected with early localized disease, amenable to R0 resection, is far from optimal, with an estimated survival no longer than 24 months (4, 44).

As aforementioned, there are many challenges that make screening and early PDAC detection a demanding task. First, due to location of the pancreas, many signs and symptoms related to tumor are only evident at an advanced stage. Second, because characteristically the progression of PDAC is usually rapid, with ability to metastasize soon after invasive disease is present (134). Supporting this, a recent report from the National Cancer Institute's Surveillance, Epidemiology, and End Results database revealed that patients with stage IV PDAC were, on average, only 1.3 years older than those with stage I disease (135).

An effective screening strategy should allow a reasonable interval of time during which potentially curable lesions could be detected and treated.

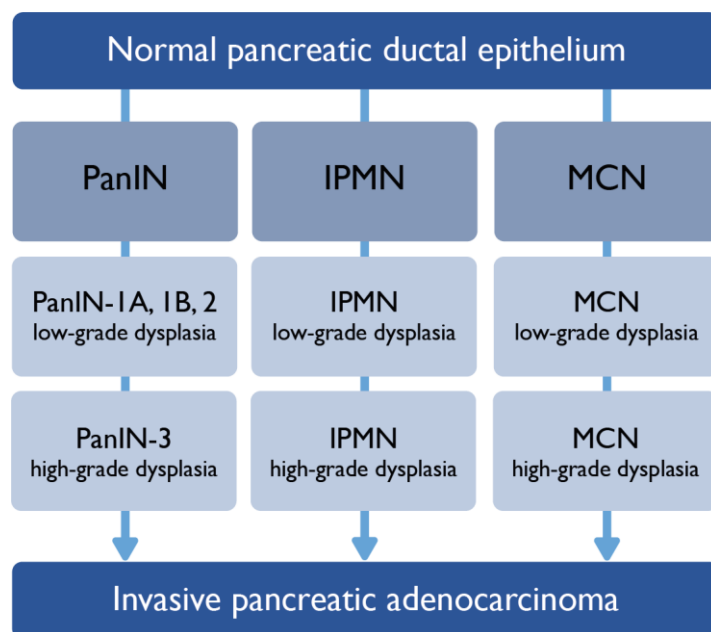
Previous research has shown that there are opportunities to explore, in order to pursue early detection of potentially curable malignant neoplasms or high-grade preneoplastic lesions (134).

The first genetic progression model from the precursor lesions termed PanIN to PDAC was reported by Hruban et al. (136). PanINs are defined as non-invasive small epithelial tumors, with different degrees of cytological and architectural atypia, located in the minor pancreatic ducts (137).

In this model, the extent of atypia was classified as PanIN-1 (low-grade dysplasia), PanIN-2 (intermediate-grade dysplasia), and PanIN-3 (high-grade dysplasia / carcinoma in situ). PanIN-1 are subdivided in PanIN-1A (flat) and PanIN-1B (papillary). PanIN-3 lesions are commonly observed in association with invasive PDAC. This classification and grading system has tremendously helped the understanding of these lesions and was subsequently incorporated in the World Health Organization (WHO) classification of tumors of the digestive system published in 2010 (138).

More recently, after the Baltimore consensus meeting for neoplastic precursor lesions in the pancreas (139), this classification has been revised and only two grades of dysplasia have been considered: PanIN-1 and PanIN-2 have been grouped as low-grade dysplastic lesions and PanIN-3 maintained the designation of high-grade dysplasia lesion/carcinoma in situ. Moreover, to improve concordance and to align with practical consequences, this 2-tiered system (low vs. high grade) was proposed for all PDAC precursor lesions, including the IPMNs and MCNs (Figure C). This concept was further accepted by WHO and is presently contemplated in its last version of the classification of tumors of the digestive system (2019) (74).

Figure C. Precursor lesions and distinct pathways to invasive PDAC.



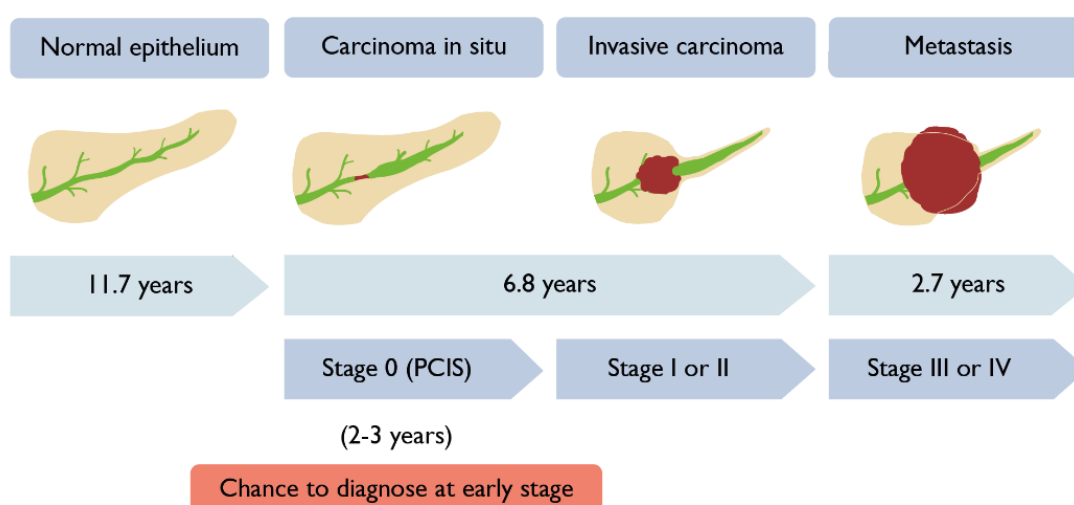
Adapted from Basturk O. et al. Am J Surg Pathol. 2015. (139)

It has been described that KRAS mutations in PanIN-1, CDKN2A/p16 inactivating mutations in PanIN-2, TP53 and SMAD4 inactivating mutations in PanIN-3 are frequently present, and this is generally accepted as the genetic model of carcinogenesis leading to invasive PDAC (140, 141).

Another hallmark study on this matter was conducted by Yachida et al. (142, 143), that estimated the lifetime of clonal evolution during PDAC development and progression based on a computational model using autopsy cases. Data was generated by sequencing the genomes to evaluate the clonal relationships among primary and metastatic cancers. Importantly, these authors showed that the driver mutations of the genes encoding KRAS, TP53, SMAD4 and CDKN2A were already present in the primary tumors and persisted in the metastatic lesions, and for this, should also be regarded as the founder mutations.

This model proposed an average of 11.7 years from the initiating carcinogenesis until development of the parental clone, an average of 6.8 years to the appearance of metastatic subclones within the primary tumor, and an average of 2.7 years until the death of the patient (Figure D). As already stated, most PDAC cases are diagnosed toward the end of this natural history lifetime, suggesting that the poor prognosis is mainly a consequence of late diagnosis (144).

Figure D. Model of progression of PDAC based in genomic sequencing studies.



Adapted from Hanada K. et al. Ann Gastroenterol Surg. 2017. (144)

These data deliver novel insights into the genetic features underlying PDAC progression. The important finding that pancreatic tumors are present for a significant period of time before clinical manifestation emphasizes the potential of use of screening and defines a broad time window of opportunity (at least of 2 to 3 years) for improving outcomes through early detection of the disease (44).

Taking all this into account, the ideal screening strategy for PDAC would target high-grade benign noninvasive precursor neoplastic lesions, before malignant transformation occurs, allowing curative intervention, such as surgical resection.

Meanwhile, two major problems subsist. First, while IPMNs and MCNs can be detected as cystic lesions or as a dilated main pancreatic duct, PanINs are not reliably visualized by clinical imaging tests. Second, it is crucial to distinguish precursor lesions with a reasonable chance of progressing to advanced cancer from those with little or no risk of progression, in order to avoid unnecessary and invasive interventions (44).

The precursor lesions identification and its stratification in terms of malignant potential is still extremely difficult, and consequently, the optimal strategy for the early detection of pancreatic neoplasia has to improve. In this process, it is our opinion that the need to involve highly accurate biomarkers should be considered, along with developments in imaging modalities.

In recent years, research has been driven by a growing enthusiasm on the identification of highly sensitive and specific biomarkers for PDAC, that should be capable of identifying the neoplastic process in its pre-invasive stage. The corollary of the successful identification of this high-grade precursor lesions, or even the cancer itself at a very early stages, would result, certainly, in a significant increase in the chance for curable treatments and an unquestionable overall gain in patients survival (145).

At present, only individuals at high hereditary risk are considered for screening, mainly based on annual MRI and EUS (58, 67). Moreover, individuals harboring MCLs (IPMNs and MCNs) are eligible for surveillance, also by MRI/magnetic resonance cholangio-pancreatography (MRCP) and EUS, in order to promptly identify changes suspicious of malignant transformation (104-108). However, these individuals represent

the minority of the spectrum of PDAC cases. The remaining sporadic cases are the major burden who should deserve special focus and interventional strategies in order to improve overall PDAC survival.

This is the challenge for the future: the search for credentialed biomarker(s) with adequate performance criteria for adoption in an asymptomatic prospective population.

3.2.2. Endoscopic ultrasound: role in PDAC and precursor lesions

EUS was first developed in the early 1980s by investigators in Japan, Germany, and the United States, mostly because of the limitations of transabdominal ultrasound in imaging the pancreas. Now, four decades later, EUS is recognized as the single best modality for detection of pancreatic tumors, with a reported sensitivity of over 95% (146, 147).

3.2.2.

1) EUS in PDAC: from diagnosis and staging to treatment

The clinical indications for diagnostic imaging in PDAC include detection of the primary tumor, assessment of its resectability, evaluation for distant metastasis, and monitoring response to treatment (147).

Abdominal ultrasound, besides its unequivocal advantages (easily available, inexpensive, safe), cannot be recommended as a premium modality for pancreatic assessment (57). In fact, its sensitivity is no more than 70% for parenchymal changes, and, more importantly, it is a technique that frequently cannot accurately evaluate the entire gland (148).

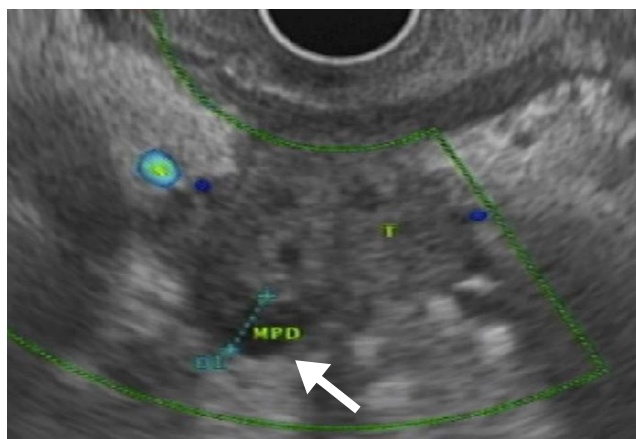
Multidetector computed tomography (MDCT), performed with a pancreatic protocol, is nowadays considered the method of choice for initial evaluation in patients with the suspicion of a pancreatic mass (149). Nevertheless, despite its high accuracy for detecting and staging pancreatic malignancies, the sensitivity of MDCT may be suboptimal in detection of lesions < 2 cm (150).

MRI has similar sensitivity and specificity, however, due to lack of wide availability, needed expertise, higher cost, and limited spatial resolution as compared to MDCT, it is not widely used as the primary imaging modality. The main role of MRI is to evaluate isoattenuated pancreatic lesions and to better characterize undetermined liver lesions identified at prior MDCT (149, 151). Recently, MRI capabilities have been improved by the addition of diffusion-weighted imaging (DWI) which brought functional aspects into conventional morphologic evaluation. This upgrade represents a promising technique to

improve characterization of pancreatic solid lesions, especially on the differentiation between PDAC and mass forming CP (152).

EUS has been achieving increasing importance in assisting in the diagnosis and staging of PDAC (Figure E). The advantages of EUS over MDCT have been reported for more than 10 years. Despite being a technique not universally accessible, operator-dependent, with inherent risks of invasiveness and sedation, EUS has progressively been considered as the most accurate tool to investigate disorders of the pancreas. It has the advantage of being a non-radiation technique that can offer high-resolution images and accurately characterize solid and cystic lesions. It has the possibility to evaluate the features of cystic wall associated with increased risk of malignancy, namely mural nodules and other focal thickenings (153, 154).

Figure E. Heterogenous, mainly hypoechogenic, solid mass, located in the head of the pancreas, causing MPD obstruction (arrow).



Adapted from Moutinho-Ribeiro P. et al. Dig Liver Dis 2019. (155)

In solid pancreatic lesions, the reported sensitivities for EUS in most studies is over 90% (156). It has a significantly better accuracy for the diagnosis of PDAC when compared to MDCT [sensitivity up to 100% for EUS vs. 86% for MDCT] (153, 154, 156, 157), being particularly exceptional for identification of lesions < 3 cm. In this setting, it presents a sensitivity of 93% in comparison to 53% for MDCT and 67% for MRI (156, 158).

In fact, EUS was shown to be able to detect lesions as small as 2-3 mm, which is generally considered the resolution of MDCT and MRI (159).

Despite this high accuracy, EUS shows some limitations, especially in the differentiating PDAC from some non-neoplastic conditions, such as autoimmune pancreatitis and mass-forming CP (155). Contrast-enhanced EUS (CEUS) and EUS-guided elastography are advances of the technique in order to improve its accuracy that have been developed to overcome some of these limitations (160). CEUS consists on the administration of a specific contrast agent, composed of microbubbles, which, during real-time evaluation, highlights the microvasculature of the pancreatic masses. PDAC is characterized by a hypovascular pattern, whereas neuroendocrine tumors show a hypervascular pattern and mass-forming CP an isovascular one. This hypoenhancement pattern has been reported to have a sensitivity of 95% and specificity of 89% in the diagnosis of PC (161-163).

EUS-guided elastography consists in the evaluation of stiffness of tissues, by measuring the degree of strain generated by external pressure, both qualitatively and quantitatively (164, 165). The qualitative evaluation is based on the color map and its pattern, while the quantitative one is based on strain ratio or strain histogram. In general, malignancy is associated with hard lesions, showing a blue pattern, high strain ratio (> 10) or low strain histogram (< 50) (160). Although its high sensitivity for determining malignancy, it still lacks specificity, and its interpretation should be taken with caution (166, 167).

Apart from being capable of improving lesion characterization, both CEUS and elastography, also have a role in the selection of the best area to perform EUS-guided tissue acquisition if indicated.

Besides elastography and CEUS, confocal laser endomicroscopy has recently appeared as a technological improvement to EUS with a particular role in cystic lesions evaluation, as previously mentioned in chapter 3.2.1.(98, 99).

Having said this, nowadays, EUS main use derives from its unique advantage of performing guided tissue FNA or FNB, during the work up of patients suspected of having a pancreatic tumor. Since the introduction of EUS-FNA in the 1990s (168), it has become a mainstay in assisting in the diagnosis and staging of PDAC (Figure F).

Figure F. EUS-guided FNA (arrow) of a pancreatic solid mass.



Adapted from Moutinho-Ribeiro P. et al. Dig Liver Dis 2019. (155)

EUS guided-FNA (EUS-FNA) or fine needle biopsy (EUS-FNB) can be performed in pancreatic masses during the procedure, to obtain tissue samples for cyto-histopathological characterization, with a low complications rate and high diagnostic accuracy (155).

In the past, the fear of tumor spread has limited its use, but recent studies have demonstrated that EUS-guided tissue acquisition is a safe procedure, with rare and mild complications, occurring in approximately 2% of the procedures (169), what has contributed to the increasing incorporation of this technique in PDAC management algorithms (59, 170-172).

Meanwhile, some controversy still exists related to the role of EUS-guided tissue acquisition in the preoperative assessment of patients presenting with promptly resectable tumors (early PC) (155).

Nevertheless, it is our believe that a biopsy proving malignancy increases the certainty of the indication of a surgical treatment, facilitating the surgeon-patient dialogue. EUS-guided tissue acquisition is also especially useful in atypical and doubtful situations and in high surgical risk patients, in whom an unnecessary surgery and its morbimortality should definitely be avoided for lesions that can mimic PC, such as autoimmune pancreatitis or lymphoma (155).

In other hand, more consensual is to perform a biopsy for unresectable and borderline resectable PC, once it is accepted as mandatory to have a cyto/histological confirmation of malignancy before treatment delivery (59, 170-172).

For solid pancreatic masses, the sensitivity (84.3%), specificity (97%), and accuracy (84%) of EUS-FNA are very high. Meanwhile, the reported negative predictive value is low (64%) (173, 174). This should be taken in consideration, especially in highly suspicious cases. In fact, a negative cyto-histological result from FNA may not exclude the presence of a (pre)malignant lesion, and, in these situations, tissue acquisition should be repeated. The use of cutting needles for EUS-FNB has been shown to improve pancreatic histological samples (175-178).

Another strategy that can be adopted to improve diagnostic accuracy is the use of rapid on-site evaluation (ROSE) of the specimen by an experienced pathologist. In fact, ROSE was found to result in fewer repeated procedures and higher percentage of definitive diagnosis (179).

Regarding EUS performance in PDAC staging, a recent meta-analysis including over 1300 patients (29 studies), revealed a pooled sensitivity and specificity of 69% and 81%, respectively, for detection of metastatic lymph nodes, and a pooled sensitivity and specificity of 85% and 91%, respectively, for vascular invasion (180). In terms of comparison with MCDT performance for staging purpose, this same analysis showed that, albeit EUS and MDCT have same ability to detect venous invasion, MDCT may be superior in assessing arterial involvement, namely the celiac axis and superior mesenteric artery (180). No significant differences between both techniques in predicting resectability were noted, with EUS revealing a pooled sensitivity of 90% and specificity of 86%. Additionally, this meta-analysis highlighted the important role of EUS-FNA in the characterization of atypical portocaval lymph nodes, peritoneal nodules, suspected liver metastasis and ascitic fluid.

For these reasons, EUS may now be considered standard of care complementing MDCT in preoperative staging of PC (180).

MRI has a minor role in this context, being almost uniquely important in accessing liver metastasis in a patient with resectable disease where MCDT showed a “clear”

hepatic parenchyma (due to the 10% rate of false negatives for the purpose of this technique (181).

Furthermore, a pancreatic protocol MRI with contrast is the preferred imaging alternative to a pancreatic protocol MDCT if a patient has an iodine contrast allergy (28).

Besides being an indispensable tool in the management of PDAC concerning diagnosis and staging, EUS has been gaining importance for its therapeutic purposes.

It is well established that, like endoscopic retrograde cholangio-pancreatography (ERCP), EUS has evolved, over the last few decades, from a diagnostic technique to a minimally invasive therapeutic alternative to radiologic and surgical procedures for pancreatic diseases, including PDAC (182).

In relation to PC management in terms of treatment, the role of EUS can be divided mainly in two actions: the handling of PC-related symptoms and the delivery of anti-tumor therapies (182).

In the former the role of EUS is now well established, namely in pain control through celiac plexus interventions (183-185), treatment of obstructive jaundice after failed ERCP via EUS-guided bilio-digestive anastomosis (186, 187), and, more recently, gastric outlet obstruction (188).

On the other hand, its use as a mean to the delivery of anti-tumor therapies (injecting anti-tumor agents, assisting in radiotherapy, and guiding ablative therapies) is still mostly experimental and have yet to evolve before becoming routine practice, despite growing evidence supporting its feasibility, safety and efficacy (189-194).

Data on the long-term efficacy of these new approaches, and large prospective randomized studies are needed to confirm its real clinical benefits.

3.2.2.

II) EUS for identification of PDAC precursor lesions in screening high-risk individuals

High-risk individuals (HRI) for PC can benefit from pancreatic screening mainly based on annual MRI and EUS (58, 67). Successful screening targets are early invasive PC and MCLs or PanIN with high grade dysplasia (HGD), which may be treated surgically with curative intent (51). These lesions are identified in 2% to 5% of all HRI undergoing screening (195, 196).

In general, many studies have suggested that EUS provides better detection of small solid lesions, while MRI, in special MRCP can be superior in characterization of small cysts, due to the possibility of imaging pancreatic ductal anatomy noninvasively (173, 197, 198).

In the classical study of the American Cancer of the Pancreas Screening (CAPS) Consortium, published in 2012 by Canto et al. (197), it was reported that in 216 HRI, pancreatic abnormalities (cysts, solid lesions or CP-like changes) were detected by EUS, MRI and MDCT in 43%, 33% and 11% of patients, respectively. This study importantly showed a sensitivity of 93% for EUS for the detection of solid lesions < 2 cm, much higher than the ones achieved by MDCT (53%) or MRI (67%).

The benefits of PDAC screening in this scenario have been recently demonstrated in a study that assessed the risk of neoplastic progression in a large cohort of 354 individuals with genetic high risk for PC undergoing long-term surveillance (24, 25). In this study, all subjects were submitted to a baseline EUS and underwent surveillance with EUS, MRI or MDCT. During the period of follow-up, lesions with worrisome features (solid mass, multiple cysts, cyst size > 3 cm, thickened/enhancing walls, mural nodule, dilated main pancreatic duct > 5 mm or abrupt change in caliber) or a rapid cyst growth (> 4 mm/year) were detected in 68 (19%) of subjects. Over a 16-year period, 7% of patients had neoplastic progression, developing either PDAC (14 cases) or high-grade dysplastic lesions (10 cases), with a rate of progression calculated of 1.6% per year. Interestingly, more than 90% of these cases had previously detected lesions with worrisome features. Another remarkable observation was that 10 out of 14 PDAC cases were diagnosed during surveillance, in completely asymptomatic patients. Nine of these 10 PDAC patients had resectable tumors and 85% of them survived 3 years or more,

compared with only 25% of the 4 individuals presenting with symptomatic, unresectable cancers.

More recently, on behalf of the Dutch Familial Pancreatic Cancer Surveillance Study Group, another important study evaluated the benefits of screening and long-term surveillance for PDAC in 366 HRI (201 mutation-negative FPC kindreds and 165 PDAC susceptibility gene mutation carriers) (25). These individuals were prospectively enrolled between 2006 and 2019 and subjected to annual surveillance with both EUS and MRI/MRCP. EUS detected more solid lesions (100% vs. 22%), but less cystic lesions (42% vs. 83%) when compared to MRI/MRCP ($p < 0.001$). During an average follow-up of 63 months, 10 individuals developed PDAC, 4 of which presented with a symptomatic non-resectable interval carcinoma and 6 localized lesions amenable for surgical resection. Cumulative PDAC incidence was 9.3% among the mutation carriers and 0% in the FPC kindreds, with a median PDAC survival of 18 months. Surgery was performed in 17 individuals (4.6%), whose pathology revealed 6 PDACs (3 T1N0M0), 7 low-grade precursor lesions, 2 neuroendocrine tumors, 1 autoimmune pancreatitis and no abnormality in one subject. The authors concluded that the diagnostic yield of PDAC was substantial in high-risk mutation carriers, but non-existent in the mutation-negative FPC kindreds. Meanwhile, timely diagnosis of resectable lesions proved challenging despite the concurrent use of EUS and MRI/MRCP. In fact, the sole use of imaging modalities revealed suboptimal results, justifying the need for more sensitive diagnostic markers, including biomarkers.

Pancreatic abnormalities in EUS screening

Pancreatic lesions can be detected by EUS screening in 19% to 79% of HRI, depending if the studies did or did not consider CP-like parenchymal changes (51).

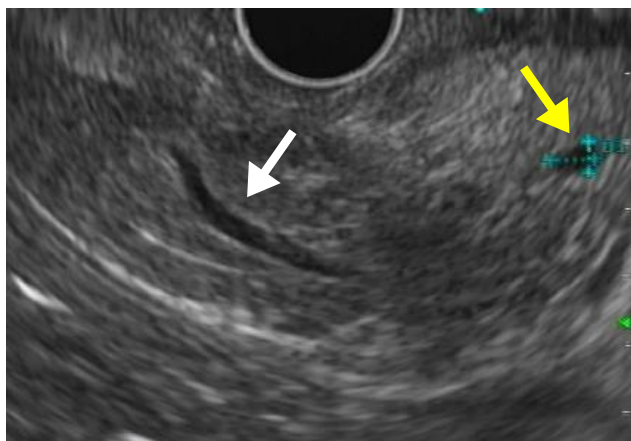
These abnormalities comprise cystic lesions, CP-like parenchymal changes, and solid lesions, which are always considered suspicious (52, 198-200).

A) Pancreatic cystic lesions

The cystic lesions more frequently identified among HRI are IPMNs, which are reported to be more frequent among these individuals (13-20%) in comparison to general population (1-5%) (200, 201). The natural history of IPMNs is not completely understood and there is no current evidence that it differs in both populations. Therefore, clinical decisions and follow-up programs in the setting of HRI should be guided by the algorithms proposed for general population.

It has to be underlined that in HRI harboring pancreatic cyst(s) (Figure G), the whole pancreatic parenchyma is at risk of developing PDAC, and not only the cyst (202, 203).

Figure G. Pancreas with a normal parenchyma presenting a 3 mm cyst in its body (yellow arrow). Main pancreatic duct with normal caliber (white arrow).



Adapted from Moutinho-Ribeiro P. et al. World J Gastroenterol. 2022.(84)

B) Chronic-pancreatitis-like parenchymal changes

One of the most used EUS criteria for diagnosing CP is based in the Rosemont classification, which takes into account both parenchymal and ductular abnormalities (204). These include major criteria: parenchymal calcifications, parenchymal lobularity

with honeycombing and main pancreatic duct calcification; and minor criteria: small cysts, parenchyma with hyperechoic foci without shadowing, hyperechoic strands, and lobularity with noncontiguous lobules, hyperechoic duct walls, irregular pancreatic ducts and dilated main duct (≥ 3.5 mm) or side branches (≥ 1 mm) (Figure H).

Figure H. Pancreatic head with CP-like parenchymal changes (lobulation, hyperechoic foci and strands).



Adapted from Moutinho-Ribeiro P. et al. World J Gastroenterol. 2022. (84)

In the context of HRI screening and PDAC precursor lesions, CP-like parenchymal atrophy and hyperechoic foci have been found to frequently correspond to multifocal PanIN lesions (205-207). These alterations are described more frequently in HRI (up to 80%) than in general population (15%-20%) (199, 208).

In 2006, a study from Brune et al. (209), including patients that underwent surgical resection, already reported a significant correlation between these changes found on EUS and the percentage of PanIN lesions on histological evaluation of surgical specimens. One of the explanations for these EUS findings is that multifocal PanIN lesions can lead to obstructive lobular atrophy (205).

It is well known that most PDAC arise from PanIN lesions, with an approximate delay of 10 years (142). The major challenge of screening is to detect these lesions at the stage of high-grade dysplasia, in order to propose pancreatectomy. Albeit some aspects of high-grade PanINs have been described with EUS and MRI, these findings lack specificity and more evidence is needed (199, 209, 210).

C) Solid pancreatic lesions

Solid pancreatic lesions can be identified by EUS in 1-6 % of HRI during follow-up (197, 198, 200).

The exact nature of these lesions has to be determined usually by EUS-FNA or even surgical resection, as they correspond mostly to PDAC, but may also be PanNET or PanIN lesions with advanced histology (173, 196, 211).

The superiority of EUS in identifying these lesions has been extensively demonstrated in several studies including patients submitted to pancreatic resection. In published reports where data from MRI and EUS were available, 26% of operated suspicious lesions were only previously detected by EUS (198, 199, 211, 212).

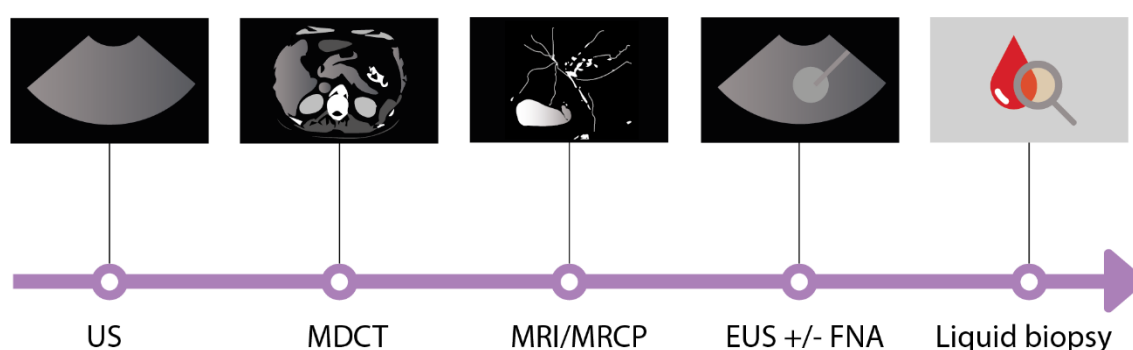
The more consensual approach to these lesions is its surgical resection, even if a decision of not to perform EUS-FNA has been made, or in the presence of a negative or inconclusive result. In fact, although knowing from previous reports that 27% of resected suspicious solid masses revealed only low-grade changes with no relevant malignant potential on histology (196), it is very risky to consider not proposing these HRI to surgical treatment.

3.2.3. Biomarkers and liquid biopsy: exosomal glypican-I, the “new kit on the block”

As stated before, early detection of PC is still very challenging, given the imperfect accuracy of available imaging methods and still the lack of a credentialed biomarker with suitable performance. The first imaging modality that appeared was abdominal ultrasonography (US) that lacks sensitivity to analyze the entire pancreatic parenchyma (Figure I). MDCT is the most used method to diagnose and stage pancreatic masses but lacks accuracy for lesions smaller than 2 cm. MRI and MRCP have important role specially in characterization of small cystic lesions and its relation to the main pancreatic duct. EUS has been assuming a gold standard position in PDAC diagnosis mostly because its accuracy in detecting very small lesions and given the possibility to perform FNA or FNB during the procedure.

Meanwhile, considerable research is being conducted in the field of liquid biopsy. This innovative concept refers to a simple and painless collection of a body fluid sample (usually blood), in order to study proved and anticipated biomarkers with the potential to identify PDAC in its earlier stages, or even better, its high-risk precursor lesions (Figure I). This will give the chance to improve the clinical management of patients and, hopefully, in a near future, it will be possible to change the paradigm of this dismal disease.

Figure I. Timeline of development of PC diagnostic tools, from imaging modalities techniques to liquid biopsy.



US: abdominal ultrasonography; MDCT: multidetector computed tomography; MRI: magnetic resonance imaging; MRCP: magnetic resonance cholangio-pancreatography; EUS: endoscopic ultrasound; FNA: fine needle aspiration

Adapted from Moutinho-Ribeiro P. et al. Front Endocrinol 2018. (145)

3.2.3.

1) Overview: current and upcoming tumor markers

The National Cancer Institute (NCI) has defined biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (213).

Biomarkers are very important in the management of patients with invasive cancers, considered essential tools for early diagnosis, prediction of therapeutic response and prognosis. Moreover, they may represent key assets in identification of a sub-group of population to target preventive interventions (133).

In PDAC, the absence of validated biomarkers contributes to patient’s poor outcome.

Hundreds of published papers reporting panels of new potential biomarkers based on biological, immunological, and genetic alterations, using blood samples and other body fluids, have been reported up to now. Nonetheless, no single candidate has been translated to clinical use for the early detection of PDAC (18).

It is assured that no biomarker currently exists with adequate sensitivity and specificity for routine clinical diagnosis or screening of PDAC (44).

The carbohydrate antigen 19-9 (CA 19-9), meanwhile, remains the only biomarker approved by the US FDA to be used in PDAC patients’ clinical management (183).

Nevertheless, it is not specific and can be found in normal pancreatic and biliary duct cells, as well as in gastric, endometrial, colonic, and salivary epithelia. CA 19-9 can be elevated in other medical conditions such as acute cholangitis, pancreatitis, obstructive jaundice and liver cirrhosis (213).

Depending on the series, its specificity ranges from 30 to 100% (214), so the extremely high rate of false positive results prohibits its routine use as a single tool for diagnosis.

Moreover, CA 19-9 sensitivity is also imperfect, ranging from 41 to 86% (214). Additionally, Lewis-negative blood type patient, which constitutes 5-10% of the caucasian population, do not produce CA 19-9, thus contributing to false negativity (215).

Another important issue is the fact that only 65% of patients with resectable tumors have increased levels of CA 19-9. Moreover, in relation to its ability to differentiate PC from CP, this biomarker is also inadequate, as up to 40% of patients harboring this last condition present CA 19-9 levels above the normal range (216, 217). Taking into account all these pitfalls, serum CA 19-9 is used primarily as a prognostic tool in monitoring patients for recurrence or managing those with late stage disease (216).

Few other carbohydrate antigens have also been studied extensively including CA-242, CA-50, CA-195, CA 72-4, CEA and CA-125, and all of them were found to be overall less sensitive than CA 19-9 (214, 218).

Considering all these aspects, it is understandable that most PDAC patients present with advanced disease, with only up to 20-25% having the possibility to be submitted to tumor resection (44). And even in these cases, it should be underlined again that most of the patients will survive at best 24 months (4, 44). Therefore, aiming to detect the “real” curable lesions, that is the very small malignant and the premalignant ones with high risk of progression, should be our main focus, along with the improvement of novel therapeutic strategies.

For early detection, the conventional diagnostic tools are far from being competent. In fact, available imaging techniques and non-biopsy tests (serum CA19-9 levels) lack the sensitivity and specificity necessary to identify very small tumors (< 1 cm) in the context of a 1.5% lifetime risk disease (219-221).

New and improved strategies which can overtake all the obstacles previously described are urgent in order to overcome the dismal prognosis of this disease.

Identifying robust biomarkers, namely for earlier detection, is essential to enable treatment of PDAC with curative intent and reduce the mortality, especially in the main group of sporadic cases. Also, in high-risk groups, they may play a role by enriching those with highest chance of developing cancer, prioritizing individuals for screening (18, 222).

In recent years, much effort has been invested in identifying accurate tumor markers, ideally present in the timeframe between cancer onset and invasion (223, 224).

Current investigation has been focused mainly on finding serum biomarkers and almost all classes of assays reported elements to be used in the setting of symptomatic disease, that is as “diagnostic biomarkers”. In the other hand, scarce studies have been made in the context of longitudinal follow-up of asymptomatic high-risk individuals, trying to discover and propose the so much desired and so-called “surveillance biomarkers” (28, 213).

For both purposes, the ideal biomarker has to be easily detected in body fluids, and present adequate sensitivity and specificity. Besides the ability to distinguish PC from benign pancreatic lesions, the identification of high-grade preneoplastic conditions, such as PanINs, IPMNs and MCNs, assumes great importance.

3.2.3.

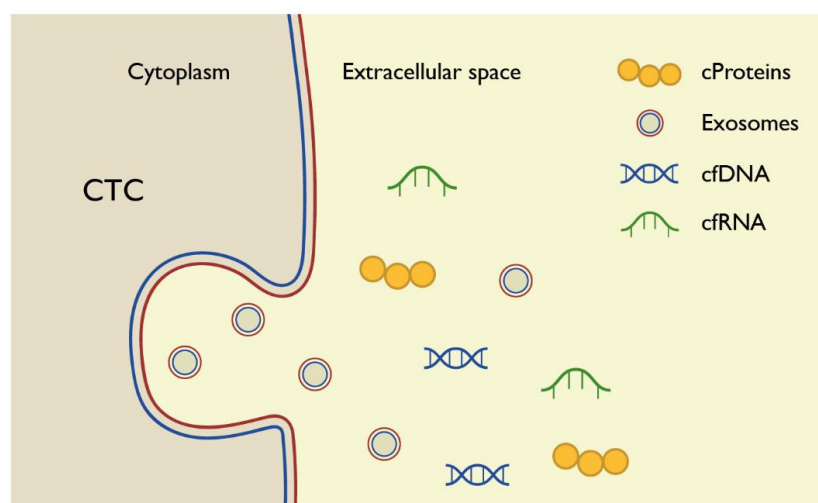
II) The new era of liquid biopsy

Liquid biopsies have gained particular interest from a clinical point of view because they represent a non-invasive tool to access biomarkers, related to primary tumours and distant metastasis, in a sample of body fluid, collected through a simple and painless technique (145, 225). They have already been implemented in the management of a number of epithelial cancer patients (226, 227).

The mostly used body fluids for biomarker assessment are the blood, urine and saliva (228-230). Blood, being easily accessible and relatively stable, is considered to be an ideal specimen to explore biomarkers. Nevertheless, biomarkers present in the serum are usually very diluted and their identification can be impaired by the presence of other more concentrated substances (231). Recent technological improvements namely based on “omics” analysis have been made to overcome this drawback, especially for isolation of nucleic acids, circulating tumor cells (CTCs), proteins and exosomes. Also, mass spectrometry-based approaches and proteomic technologies have been useful for the analysis of low abundance proteins (232).

A myriad of circulating elements may be used as tumor markers, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), CTCs, circulating tumor proteins, and extracellular vesicles, more specifically exosomes (Figure J) (222, 224).

Figure J. Molecular biomarkers for PC: cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTC), circulating tumor proteins, and extracellular vesicles, including exosomes.



Adapted from Moutinho-Ribeiro P. et al. *Front Endocrinol* 2018. (145)

The recent possibility to pair genomic tests with analysis of CTCs, circulating tumor nucleic acids (ctNAs) and tumor-derived exosomes, raised the importance of liquid biopsy in terms of applications for clinical use (229, 233, 234).

In the particular case of PC, several genetic modifications occur during the initiation and progression processes. These include genetic diversification, amplifications and homozygous deletions, an increase in duplicate chromosomal number, recapitulation of clonal expansion, clonal selection, driver mutations and losses of heterozygosity (142, 235-238).

Next-generation sequencing (NGS) techniques have led to deeper insight into somatic mutations and epigenetic analysis, allowing a better characterization of circulating tumor DNA and RNA (ctDNA, ctRNA) (239). The development of cell tracking techniques and flow cytometry permitted the capture and analysis of CTCs and exosomes (232).

For all these reasons, the potential of liquid biopsy is vast, as a means of identification of tumor biomarkers in a similar way of conventional tissue biopsy, contributing to the characterization of tumor heterogeneity, and, ultimately, to cancer early detection, monitoring disease progression and response to therapy (229, 234, 240-242).

A recent systematic review and meta-analysis of 19 studies, including 1872 individuals, confirmed the overall high diagnostic value of various liquid biopsy methods in detecting PC, namely ctDNA, CTCs and exosomes. Among these three biomarkers, exosomes showed the highest sensitivity and specificity (243).

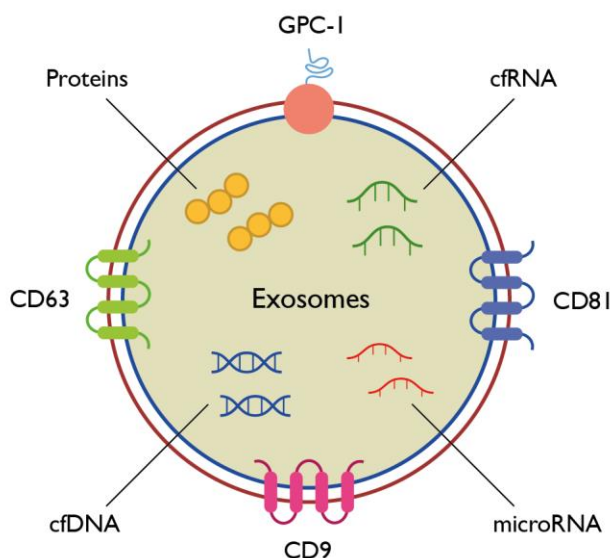
3.2.3.

III) Exosomes, premium biomarkers?

Over the last years we have observed an increasing interest in the field of exosomes. Although much is still unknown about their role in human health and disease, different disciplines are recognizing the highly conserved role that exosomes play in important biological processes, namely in cancer biology (244).

Exosomes are small cup-shaped nanovesicles (50–150 nm in size) produced by nearly every human cell type, including cancer cells (245). Structurally, they are constituted by a lipid bilayer membrane with tissue-specific content, such as mRNA (messenger RNA), miRNA, DNA fragments, and proteins (246, 247). The surface markers CD63, CD9 and CD81 are recognized to be generally expressed in exosomes and are regularly used as exosomal biomarkers (Figure K) (246, 248). After secretion, exosomes are stable in the extracellular environment or enter the circulation. They can be uptaken by neighbor or distant cells and allow the exchange of material and information between cells, modifying gene expression or mediating RNA silencing (249-251).

Figure K. Schematic representation of exosomes.

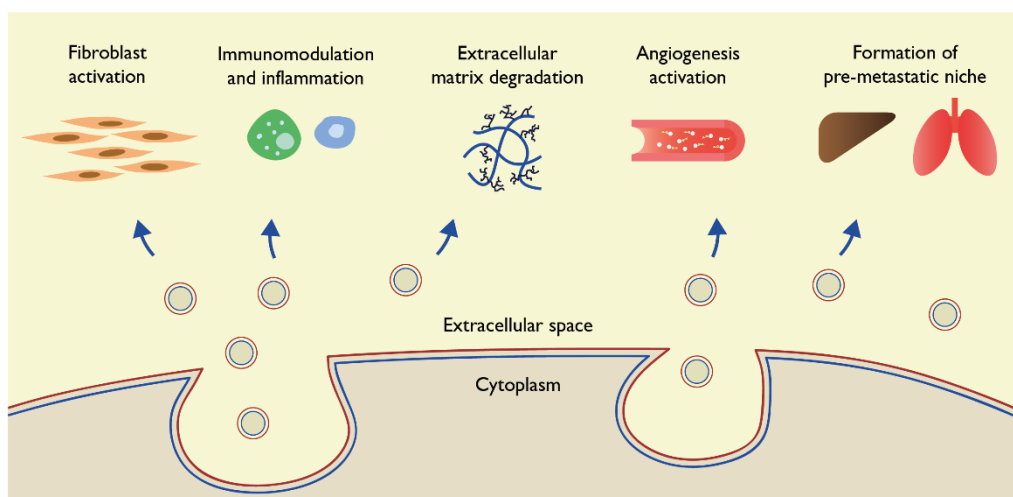


Adapted from Ruivo CF. et al. Springer International Publishing AG 2018. (248)

Cancer cells exhibit abnormal exosomes physiology (252). When compared with nonmalignant cells, malignant cells secrete greater amounts of exosomes, contain higher quantities of miRNAs, and contain miRNAs associated with the RNA induced silencing

complex, essential for silencing of mRNAs in target cells. In fact, they have been implicated in a powerful mechanism through which cancer cells influence behaviors of target cells, both in the tumor microenvironment and in distant locations (Figure L) (13, 246, 248, 252-255).

Figure L. Schematic representation of exomes-mediated communication in cancer.



Adapted from Ruivo CF. et al. Springer International Publishing AG 2018. (248)

Their important role has been demonstrated at the level of tumorigenesis, local invasion, migration, angiogenesis, metastatic niche formation, immune evasion, and therapeutic resistance (13, 249, 252, 253, 256-259).

Understanding the way exosomes can influence tumor initiation, proliferation and metastasis is improving the knowledge on PC pathophysiology and, ultimately constitutes a hope to improve patients' prognosis.

Exosomes have been proving to be reliable candidates as PDAC biomarkers, as their contents, namely DNA, RNAs, proteins, lipids, and metabolites are mainly derived from tumor cells. Considering their extensive distribution and functions, they are considered ideal to investigate circulating biomarkers for early PDAC detection, monitoring disease progression and patient management (223, 251, 260). Moreover, due to their excellent biodistribution, biocompatibility and cell-specific nature, exosomes are believed to be used, in the future, as drug delivery vehicles (261, 262). In fact, exosomes may represent an excellent system to manipulate in the persistent effort to find more effective therapies

for PDAC. In this field, there are many potential opportunities ranging from inhibiting their biosynthesis and release by PDAC cells, to alteration of their contents and blockade of their uptake by target cells (244).

In comparison with other biomarkers, exosomes display several advantages (260, 263, 264):

First, their distribution is practically universal in all body fluids, including blood, and they can be stored at -80°C , maintaining stability for long term.

Second, as previously mentioned, cancer cells secrete more exosomes than normal ones, which can be explained, at least in part, by the facilitation of this process in the presence of acidic conditions, like the ones observed within tumor microenvironment (265).

Third, exosomes content is plural, including a combination of DNA, coding and non-coding RNAs, proteins and lipids. This diversification allows, in the same setting, simultaneous evaluation of a panel of potential biomarkers.

Fourth, and especially related to PDAC-derived exosomes, a very important feature is that exosomes are secreted into circulation during the initial phase of tumorigenesis, at very early stages of the disease and, because of that, they can be potentially used for early detection (260, 263, 264). This phenomenon is not usually seen in other cancer biomarkers, that are frequently released into the blood only after cell necrosis, normally associated with large tumor mass and advanced stages (222, 240, 263).

In terms of PDAC biology, recent studies have implicated exosomes in the early development of the tumor, promoting cancer formation and proliferation (247, 249, 260, 264, 266, 267), and they have also demonstrated their contribution for the establishment of a premetastatic niche in hepatic parenchyma (257, 258).

Additionally, there is accumulated evidence of the role of exosomes in the development of drug resistance, as well as some paraneoplastic conditions, such as cachexia and DM, which can provide some light for specific future therapeutic targets (121, 259, 268, 269).

Considering exosomes' content, proteomic profiling has been used to determine its cellular origin (270). RNA and miRNAs have been extensively investigated, essentially

because of the proven recognition of some of these molecules in cancer detection, but also justified by the relatively ease technical feasibility for its amplification (252, 271-276).

MicroRNAs belong to a class of non-coding RNA that are involved in post-transcriptional regulatory mechanisms. In PDAC, miRNAs dysregulation has been profiled in pancreatic tissue, blood and other body fluids (277-279).

In the last years, we assisted to a huge number of exosomal miRNAs being evaluated to access its potential role in PDAC early detection and patients' management. Some examples of these molecules with established importance are miR-21, miR-17-5p, miR-155, miR-34, miR-196a, miR-181a, miR-181b, miR-138-5p, miR-494, miR-542-3p, miR-31, and miR-205. In a global observation, dedicated studies have shown that these miRNAs, when upregulated, have the capacity to promote cellular proliferation, neoangiogenesis, disease progression and metastasis. They have also been implicated in resistance to chemotherapy in PC patients (273, 278-287).

Besides the relevance of exosomal miRNAs as diagnostic and prognostic biomarkers, these studies suggest the interest for their potential use as therapeutic targets in the near future. This assumes great importance particularly when dealing with a tumor with such difficulties in therapy delivery, in consequence of its dense stroma and intrinsic microenvironment resistance (288).

It must be emphasized that the great majority of investigation regarding the role of exosomes as PDAC biomarkers has been done in patients with confirmed diagnosis of cancer. Meanwhile, and always centered in the principle that when facing a dismal disease like this one, the earlier the detection, the better the chance for cure, we believe that the assessment of the performance of these biomarkers in the context of the screening and/or surveillance of high-risk individuals would be of major interest.

3.2.3.

IV) Glypican-I, the “new kit on the block”

Glypican-I (GPCI) and other glypicans belong to the family of heparan sulfate proteoglycans (HSPG) and can be detectable in blood as potential clinical biomarkers (289-291).

In mammals, it has been described GPC I through-6 (GPC6), differing in the number of the heparan sulfate (HS) chains and the protein attaching site. These proteins are located on the cell membrane, anchored by glycosylphosphatidylinositol (GPI) which is cleavable by the enzyme lipase Notum (292).

GPCI is composed of a protein backbone (558 amino acids) with the attachment of 3 HS chains and has both a membrane-anchored and a secreted soluble form (293).

It is expressed in most tissues in adults (293) and like other HSPGs and glypicans, GPCI is known to regulate tumor growth, invasion, metastasis and progression, through modulation of tumor cell biology as well as influence on the tumor microenvironment (294, 295). It mediates signaling in cell proliferation, differentiation, and organ development by interacting with cell membrane receptors via its HS side chains, including TGF- β , fibroblast growth factor (FGF) and vascular endothelial growth factor, among others (296, 297).

Besides cancer, it has been related to neurodegenerative processes, such as Alzheimer disease (298).

It is in the field of cancer that GPCI has been increasingly looked for, as it has been found to be associated with some carcinomas, namely pancreatic, breast, ovarian, prostatic, esophageal and colorectal cancer (299-306).

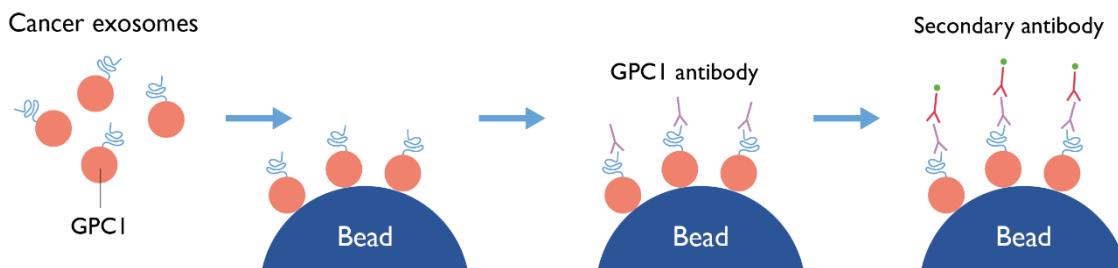
Overexpression of GPCI in PC tissue has been associated with an unfavorable prognosis and decreased patients' survival (300, 307).

Possible mechanisms of GPCI expression in cancer might involve miRNA expression and DNA hypomethylation (296).

In 2015, from the 48 proteins identified in the exosomes derived from PC tissue by mass spectrometry and bioinformatics analysis, Melo et al. (1) first reported that GPCI could be used as a marker of this cancer, with subsequent detection of GPCI in circulating exosomes (GPCI+ crExos). In this novel approach, GPCI+ crExos were

monitored with flow cytometry in serum of patients with PC, PC precancerous lesions, benign pancreatic diseases (BPDs) and healthy controls (Figure M).

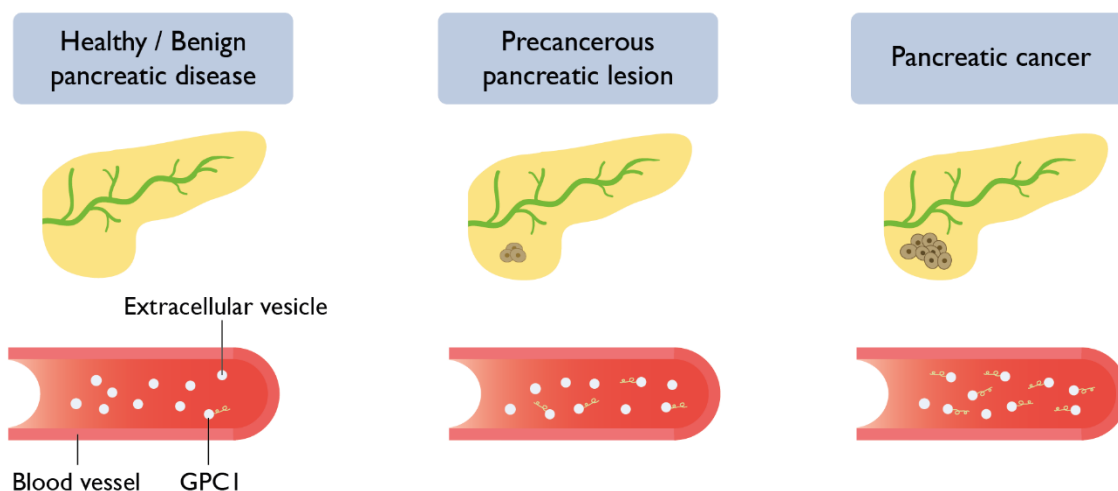
Figure M. GPC1 + crExos monitoring and isolation using fluorescence-activated cell sorting (FACS).



Adapted from Melo S. et al. Nature 2015. (1)

In the PDAC group, all 190 patients had higher levels of GPC1 + crExos than healthy individuals, with an absolute diagnostic accuracy (100% sensitivity and 100% specificity). Moreover, higher levels were also seen in both PC and PC precursor lesions, such as IPMNs, when compared to CP patients and healthy controls, with a perfect area under the curve (AUC) of 1.0. Given the high sensitivity and specificity of GPC1 + crExos in differentiating PC, independently of its stage, from non-PC controls, the authors concluded that this biomarker could have a promising role in PC early detection (Figure N). When compared to CA 19-9 (AUC: ranging between 0.69 and 0.79), exosomal GPC1 was found to be significantly superior ($p < 0.001$).

Figure N. GPC1 + crExos expression in healthy controls and benign pancreatic diseases (left), precancerous pancreatic lesions (center) and PDAC patients (right).



Adapted from Melo S. et al. Nature 2015. (1)

Another interesting point of this study resulted in the observation that GPC I + crExos exhibited positive results prior to pancreatic lesions being detectable by MRI, in a genetically engineered mouse that spontaneously developed PC. Also, in these settings, tumor burden was positively associated with levels of GPC I + crExos. Interestingly, exosomes levels reduced following the removal of the solid tumor in most of the subjects, supporting the idea that GPC I + crExos may also be seen as a prognostic indicator, reflecting tumor load and monitoring disease progression. In fact, the Cox multivariate regression analysis, recognized this marker as an independent prognostic factor for disease-specific survival. Finally, of note, in this study, direct analysis of GPC I in serum, by enzyme-linked immunosorbent assay (ELISA), presented much lower sensitivity and specificity than the quantification of GPC I in purified serum exosomes.

Many other groups since then, although utilizing different methodologies, have reported findings related to GPC I detection in exosomes in PDAC patients.

Lewis et al. (308) developed a method integrating the capture and analysis of GPC I + exosomes onto an electrokinetic microarray chip, which distinguished 20 PDAC patient samples from 11 healthy subjects, with 99% sensitivity and 82% specificity.

Qian et al. (309) isolated serum extracellular vesicles (EVs) and found that the levels of GPC I + EVs was significantly higher in patients with advanced PDAC than those in healthy controls ($p < 0.01$), and that these levels decreased following regional intra-arterial chemotherapy.

Yang et al. (310) used an advanced multiplexed plasmonic assay to analyze circulating tumor-derived EVs and identified a signature of GPC I and other four markers for PDAC detection. While the whole panel revealed a diagnostic accuracy of 84%, with a sensitivity of 86% and a specificity of 81%, GPC I alone was considered one of the best markers, with a diagnostic sensitivity and specificity of 82% and 52%, respectively.

Buscail et al. (311) conducted a prospective study to quantify GPC I + exosomes in peripheral and portal blood from PDAC patients undergoing up-front surgery, comparing to controls (patients without cancer history and patients displaying pancreatic preneoplastic lesions). Exosomes were isolated with anti-CD63 coupled magnetic beads, and GPC I positivity was assessed with the same antibody used by Melo et al. (1). GPC I positive beads were significantly higher in PDAC than in the control group, with a

diagnosis accuracy of 78% (sensitivity 64% and specificity 90%), when results from peripheral and portal blood were combined.

More recently, Xiao et al. (312), in addition to supporting Melo's findings, developed a panel consisting of exosomal GPC1, exosomal CD82, and serum CA 19-9 for PC detection. This panel was able to effectively distinguish with excellent diagnostic results (AUC = 0.942; 95% CI: 0.882-1.000) patients with PC from healthy people.

Meanwhile, different results regarding the performance of exosomal GPC1 have been reported in other studies.

In a report by Zhou et al. (313), high GPC1 serum level measured by ELISA was associated with poor prognosis, but could not be used as a marker for the clinical diagnosis of PDAC. Of note, GPC1 ELISA had been previously described by Melo et al. to be a poor technique to study this biomarker (1).

Lai et al. (314) reported that the exosomal GPC1 plasma, quantified by liquid chromatography-mass spectrometry, did not differentiate PDAC patients from controls (health subjects and CP patients). Instead, this purpose was achieved with a panel of 5 miRNAs.

In the same way, Frampton et al. (315) were unable to distinguish PDAC from BPD using ELISA in ultracentrifuged plasma. Again here, GPC1 ELISA had been previously described by Melo et al. to be a poor technique to study this biomarker (1).

Even more recently, Lucien et al. (316) measured the GPC1+ EVs, solely or in conjunction with glycoprotein 2, and found that they could not distinguish PDAC from BPDs. In this study, direct EVs detection was performed with a distinct and not established technique (nanoscale flow cytometer) and an anti-GPC1 antibody different from Melo et al. (1) study.

Several reasons may account for these different results in terms of diagnostic accuracy of GPC1 positive exosomes (296). First, discrepancies might originate from different technical approaches to isolate and measure GPC1+ crExos levels. Second, GPC1 expressed from the cancerous tissue can be confounded by its normal secretions from other tissues, once GPC1 is widely expressed in brain, gastrointestinal tract, urinary, and reproductive systems (317). Additionally, in many of the above-mentioned studies,

the specificity of the GPC I antibody was not validated, which can lead to false results (318). Also, few studies have taken in consideration the relationship between the serum GPC I levels and the cancer burden and the percentage of GPC I in cells. Another issue relates to the fact that some studies used EVs of particular sizes for the analysis, remaining questionable whether these EVs represent the expression of GPC I without bias (296). Finally, and as mentioned before, the release of GPC I from the membranes is dependent on the activity of the protease Notum (292). This enzyme may not have the same activity or be expressed in normal amount in malignant tissues. Moreover, the studies that used serum as sample, could have been influenced by the fact that, differently from plasma, serum misses fibrinogen and other components, but significantly contains large quantities of clotting factors, exhibiting highly protease activity, with possible cleavage of GPC I, thus conducting to false results (296).

In summary, GPC I is a new glypican member that has extensively been demonstrated to be increased in PDAC.

Despite few controversial observations, GPC I deserves further investigation assessing its role as potential biomarker for PDAC diagnosis and prognosis. Moreover, as it seems to be overexpressed in some of PC precursor lesions, such as IPMNs (1, 319), its potential contribution to individualize these patients' management has been recently suggested by our group (109, 110, 113).

Also, promising experimental reports have addressed the role of GPC I targeted nanocarriers, to serve as MRI contrast agents, as a part of multimodal imaging techniques, contributing for early PC diagnosis (320, 321).

Finally, and given that membrane and extracellular proteins are theoretically more accessible, GPC I will certainly continue to be a hot topic in the research field of target therapies (322-327).

3.3. REVIEW ARTICLES

To highlight the background of this thesis and to support the issues covered in the investigational project and corresponding original research papers, in the review articles we addressed:

- a) The facts related to PDAC burden and its associated risk conditions; the relevance of screening and/or early diagnosis; its precursor lesions and the window of opportunity for early management - **Paper 1.**

Moutinho-Ribeiro P, Coelho R, Giovannini M, Macedo G.

Pancreatic cancer screening: Still a delusion?

Pancreatology. 2017 Sep-Oct;17(5):754-765.

DOI: 10.1016/j.pan.2017.07.001. Epub 2017 Jul 13.

- b) The importance of EUS in PDAC management, namely as a tool for diagnosis and staging and the role of tissue acquisition techniques - **Paper 2.**

Moutinho-Ribeiro P, Iglesias-Garcia J, Gaspar R, Macedo G.

Early pancreatic cancer - The role of endoscopic ultrasound with or without tissue acquisition in diagnosis and staging.

Dig Liver Dis. 2019 Jan;51(1):4-9.

DOI: 10.1016/j.dld.2018.09.027. Epub 2018 Oct 5.

- c) The current and emerging roles of therapeutic EUS in the management of patients with PDAC: a) the established evidence for its use in the management of PDAC-related symptoms; b) the growing data supporting its feasibility, safety and efficacy, albeit mostly in experimental scenarios, as a means to the delivery of anti-tumor therapies (injection of anti-tumor agents, assisting in radiotherapy, and guiding ablative modalities) - **Paper 3.**

Moutinho-Ribeiro P, Liberal R, Macedo G.

Endoscopic ultrasound in pancreatic cancer treatment: Facts and hopes.

Clin Res Hepatol Gastroenterol. 2019 Oct;43(5):513-521.

DOI: 10.1016/j.clinre.2019.02.014. Epub 2019 Mar 29.

- d) The concept of liquid biopsy and biomarkers, its potential to identify pre-neoplastic disease or very early stages of cancer, representing a promise to improve patient survival. In more detail, the role of exosomes in PDAC: from initiation to tumor progression and its applicability in early detection and PDAC treatment; Glypican-I as being one of the most promising exosomal associated biomarkers related to PDAC management - **Paper 4.**

Moutinho-Ribeiro P, Macedo G, Melo SA.

Pancreatic cancer diagnosis and management: Has the time come to prick the bubble?

Front Endocrinol (Lausanne). 2019 Jan 8;9:779.

DOI: 10.3389/fendo.2018.00779. eCollection 2018.

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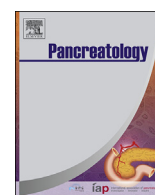
PAPER I

Moutinho-Ribeiro P, Coelho R, Giovannini M, Macedo G.

Pancreatic cancer screening: Still a delusion?

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Pancreatic cancer screening: Still a delusion?



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ABSTRACT

Pancreatic adenocarcinoma represents the fourth most common cause of cancer mortality and death due to pancreatic cancer (PC) have increased since 2003. Its incidence has also raised about 30% in the past decade and it is expected to become the second cause of cancer mortality by 2020 in the USA. Most PC present with metastatic disease and improvements in treatment outcomes for this group have been disappointing. These observations support the idea that screening to identify patients at an earlier stage might be an important strategy in improving overall PC outcomes. Many protocols have been tested, nevertheless, by now there is no effective screening program.

Given the overall low incidence of disease and the current lack of accurate, inexpensive and noninvasive screening tests, the consensus is that widespread population-based screening for PC in the general population or in patients with only one affected first-degree relative is neither practicable nor indicated in most countries. However, a different scenario is screening patients with higher risk for PC, most of them with hereditary conditions predisposing the development of this neoplasia. In fact, some guidelines are now available helping to select these individuals at risk and to screen them, in order to achieve early detection of PC.

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Introduction: the facts

Pancreatic ductal adenocarcinoma (PDAC) represents only 3% of estimated new cancer cases each year, nevertheless it is the fourth most common cause of cancer mortality with 48,960 new cases and 40,560 deaths estimated in 2015 [1]. As a matter of fact, contrary to the death rates for other leading causes of cancer death (lung, colorectal, breast and prostate), which have declined since 2003, the death rate from PDAC has increased during the same time period [2]. Its incidence has also raised about 30% in the past decade and it is expected to become the second cause of cancer mortality by 2020 in the USA [1].

As pancreatic cancer (PC) typically develops with few symptoms, only 10–20% of the patients are diagnosed at a stage amenable to resection, the only possibility of cure. Therefore, it has a poor overall five-year survival rate of 5% combining all stages, with a survival rate of about 20% and 2% for patients with localized disease and with distant metastases, respectively [3].

Over the last decades, there have been remarkable improvements in medical and cancer care. Nonetheless, these advances have only had a small beneficial impact for PC patients. The one-year survival rate for all stages has increased from 15% (between 1975 and 1979) to 21% in 2013 [4]; and five-year survival has increased from 2.5% (between 1975 and 1979) to only 7.2% (between 2005 and 2011) [4].

These survival improvements have been attributed mainly to an increased use of axial imaging techniques together with a decrease in surgical morbidity and mortality rates [5]. Patients with PDAC discovered by chance through imaging (pancreatic incidentalomas) appear to have increased survival rates. Winter et al. [6] depicted that patients with pancreatic incidentalomas had a median survival of 30 months vs. 21 months in those with carcinoma found after symptoms appearance, corroborating the idea that earlier diagnosis can lead to an improved outcome. Unfortunately, few cases of PC are diagnosed by chance. In one study during 8 years of follow-up, 24 out of 321 patients (7%) with a solid pancreatic mass had their diagnosis performed incidentally, and 58% of them were adenocarcinomas [7].

Most PC present with metastatic disease and improvements in treatment outcomes for this group have been disappointing.

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Abbreviations

PC	pancreatic cancer
PDAC	pancreatic ductal adenocarcinoma
FDR	first-degree relative
PanIN	pancreatic intraepithelial neoplasia
IPMN	intraductal papillary mucinous neoplasm
PJS	Peutz–Jeghers syndrome
EUS	endoscopic ultrasound
CT	computed tomography
MRI	magnetic resonance imaging
ERCP	endoscopic retrograde cholangio-pancreatography
PET	positron emission tomography
MRCP	magnetic resonance cholangio-pancreatography
HRIs	high-risk individuals
CA 19-9	carbohydrate antigen 19-9

MCN	mucinous cystic neoplasm
MD-IPMN	main duct intraductal papillary mucinous neoplasm
BD-IPMN	branch duct intraductal papillary mucinous neoplasm
FPC	familial pancreatic cancer
HBOC	hereditary breast and ovarian cancer syndrome
DM	diabetes mellitus
MCDT	multidetector computed tomography
MiRNA	microRNA
GPC1	glypican-1
CrExos	circulating exosomes
FAMMM	familial atypical multiple mole melanoma
DWI	diffusion-weighted imaging
MMR	mismatch repair
CAPS	Cancer of the Pancreas Screening
CEUS	contrast-enhanced ultrasound
CEA	carcinoembryonic antigen

Patients with advanced stage tumors have been typically treated with gemcitabine, which increases the median survival to 6 months from an untreated median survival of 3 months [8,9]. Numerous randomized controlled trials studying outcomes with novel chemotherapy agents and combinations (such as FOLFIRINOX) with good biologic rationale have been recently published, but median survival has not increased substantially beyond 6 months [8,10–20]. It is remarkable that with substantial effort and commitment to clinical trials, little has been gained in overall survival in metastatic PC. Regarding adjuvant treatment after resection of PDAC, a recent multicentre, open-label, phase 3 randomized clinical trial (ESPAC-4 trial) showed that the combination of gemcitabine and capecitabine was superior to gemcitabine on monotherapy and should be the new standard of care in this scenario [21].

These observations support the idea that screening to identify patients at an earlier stage might be an important strategy in improving overall PC outcomes. Many protocols have been tested, although, at this time, there is no recognized effective screening program.

The great majority of PCs, at least 90%, are considered sporadic [22–24]. Bearing in mind that PC is a rare disease, the detection rate in average risk population is low. As a matter of fact, in a screening study that included 2511 patients, only 5 cancers were detected by magnetic resonance imaging (MRI) combined with US (a detection rate of 0.20%) [25].

Given the overall low incidence of disease and the current lack of accurate, inexpensive and noninvasive screening tests, the consensus is that widespread population-based screening for PC in the general population or in patients with only one affected first-degree relative (FDR) is neither practicable nor indicated in most countries [26].

However, a different scenario is screening patients with higher risk for PC, most of them with hereditary conditions predisposing the development of this neoplasia. In fact, some guidelines are now available helping to select these individuals at risk and to screen them, in order to achieve early detection of PC [27]. Unfortunately, these individuals represent only a minority of all PCs, being the burden of sporadic cases the true enemy that should be intensively studied to help physicians reduce PC-related mortality in a near future [5].

Natural history: a window of opportunity for early detection?

PC typically develops with few symptoms that vary according to

tumor location. When symptoms are present, a characteristic pattern of painless jaundice is often recognized. However, commonly atypical patterns of symptoms such as weight loss, abdominal pain and malaise might lead to delays in diagnosis. Also, most patients present with metastatic disease, when treatment is disappointing. On the other hand, many patients initially thought to have localized and resectable cancer (10–20%) will die from recurrent or metastatic disease [5]. Despite all these adverse facts, whether the dismal prognosis of patients with PC compared to those with other types of cancer is a result of late diagnosis or early dissemination of the disease, is still unknown.

A recent study from Yachida et al. [28] suggests that there may be a large window of opportunity in the natural history of PC for its detection while the disease is in its earliest and treatable stages. In this study, genomic sequencing was performed on cancer cells obtained at autopsy from seven PC patients. Data was generated by sequencing the genomes to evaluate the clonal relationships among primary and metastatic cancers. The authors performed a quantitative analysis of the timing of the genetic evolution of PC, concluding that at least a decade between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell is necessary. Yachida et al. [28] based on the differential accumulation of mutations in primary and metastatic lesions estimated an average of 11.7 years elapsed from tumor initiation to overt cancer development and calculated an average of 6.8 years elapsed between the development of overt cancer and the development of metastatic ability. This finding that pancreatic tumors are present for a significant period of time before clinical manifestation emphasizes the potential of screening for early detection of PC.

All these data provide novel insights into the genetic features underlying PC progression and define a broad time window of opportunity for improving outcomes through identification of the disease, when treatments are likely to have a benefit, assuming suitable biomarkers can be found that correspond to the pre-cancerous or pre-metastatic time periods.

Screening goals

The ultimate goal of a cancer screening program is to improve survival rates. PC patients detected in early localized stage (stage I), amenable to R0 resection, have an estimated survival no longer than 24 months. Taking this into account, the ideal screening strategy for PC would target high-grade benign noninvasive precursor neoplastic lesions, such as pancreatic intraepithelial

neoplasias (PanINs) and the mucin producing cystic lesions, namely intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs), before malignant transformation occurs, allowing curative surgical resection [26,29]. IPMNs are grossly visible mucin producing epithelial neoplasms arising from the main or side branches pancreatic ducts [29,30]. MCNs are defined as mucin-producing and septated cyst-forming epithelial neoplasia of the pancreas with a distinctive ovarian-type stroma. Usually solitary, with a thick fibrotic wall and without communication with the ductal system [31]. MCNs are rare and, in most series, less common than IPMNs. PanIN lesions are microscopic, noninvasive neoplasms involving ducts <5–10 mm and are characterized by columnar to cuboidal cells with a varying degree of atypia [32].

Although IPMNs and MCNs can be detected as cystic lesions or as a dilated main pancreatic duct, PanINs are not reliably visualized by clinical imaging tests. The identification of these lesions and its stratification in malignant potential behavior is still extremely difficult, and hence, the optimal strategy for detection early pancreatic neoplasia has to improve and may need to involve highly sensitive and specific biomarkers, alone or in combination with imaging tests.

Recently, it was described with excellent agreement that, to be successful, a screening program should detect and treat T1N0M0 margin-negative PC and high-grade dysplastic precursor lesions (PanINs, IPMNs and MCNs) [27].

The pancreatic cancer spectrum: sporadic and associated with genetic predisposition

While most PDAC are sporadic, up to 10% may be due to an underlying genetic predisposition [33], including familial pancreatic cancer (FPC) in 7% and cases associated with hereditary cancer syndromes in 3%. FPC is considered in families with at least 2 first degree relatives (FDR) with PC not associated with another described familial hereditary cancer syndrome. In this setting, germline mutations in hereditary cancer genes (*BRCA1*, *BRCA2*, *CDKN2A*, *PALB2*) are identified in only a minority of families (nearly 20%) [34,35].

Family history was first recognized as a risk factor for PC by Henry Lynch in 1967 when PC was reported in an adenocarcinoma-prone family [36]. After that, several case-control and prospective cohort studies, have confirmed an increased risk of PC among individuals with a family history of PC [37–39]. In a prospective follow-up of the National Familial Pancreas Tumor Registry (NFPT), involving 5179 individuals (270 FPC kindreds and 468 sporadic PC kindreds), Klein et al. [40] estimated the relative risk of the development of pancreatic cancer in FPC kindreds to be 6.4 (95% CI: 1.8–16.4) and 32.0 (95% CI, 10.2–74.7) for individual with two or three affected FDR, respectively. This finding suggests that a highly penetrant gene or genes may be responsible for the familial aggregation of PC in kindreds in which multiple family members have PC.

There have been significant advances in our understanding of hereditary PC over the past decade. At present, there are known inherited cancer syndromes that predispose to PC. Inherited gene mutations as seen in hereditary pancreatitis and in inherited cancer syndromes, including: Peutz–Jeghers syndrome (PJS), Lynch syndrome, hereditary breast and ovarian cancer syndrome (HBOC) and familial atypical multiple mole melanoma (FAMMM) contribute to the familial aggregation of PCs [30]. Applying screening strategies to patients with one or more of these risk factors could help increase the performance of a putative screening test.

PJS is an autosomal dominant, hamartomatous polyposis syndrome with high penetrance caused by inherited mutations in the *STK11/LKB1* gene located at 19p13.3 [41]. A clinical diagnosis of PJS

is performed in patients with 2 of the following criteria: 2 Peutz–Jeghers polyps of the small bowel, characteristic mucocutaneous pigmentation, or a family history of PJS [30]. Patients with PJS are known to be at increased risk for common gastrointestinal and non-gastrointestinal cancers. Giardiello et al. [42] estimated that the relative risk of PC among PJS patients was 132 (CI 95% 44–261), with a lifetime cumulative risk to age 65–70 of 11–36% [43].

Lynch syndrome is an autosomal dominant inherited disorder caused by germline mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* [44]. Estimates of carrier frequency of germline mutations of these genes in the population range widely depending on various assumptions, from approximately one in 3010 individuals (for *MLH1* and *MSH2* combined) to one in 360 individuals (for all four MMR genes combined) [45]. Patients with Lynch syndrome have an estimated lifetime risk of 3.7% of developing PC (8.6-fold higher risk) [44].

HBOC syndrome is characterized by the presence of germline mutations in two cancer susceptibility genes, *BRCA1* and *BRCA2*. *BRCA1* and *BRCA2* mutation carriers have a significantly increased lifetime risk for developing breast and ovarian cancer, as high as 84% and 39% respectively [46]. *BRCA2* mutations are found in as many as 12–17% of patients with FPC and can be present even in the absence of breast or ovarian cancer, and in apparently sporadic PC [47]. Another associated inheritable gene mutation has been recently described, partner and localizer for breast cancer 2 gene (*PALB2*), as a PDAC susceptibility gene, which may be causative for 3%–4% of FPC [48]. *BRCA1* mutation-positive families had a risk for PC 2- to 3-fold greater comparing with general population. Germline *BRCA2* mutations have been clearly associated with an increased risk of PC with a relative risk of 3.51 (1.87–6.58) [30].

FAMMM is an autosomal dominantly inherited syndrome with incomplete penetrance. Germline mutations in the *p16/CDKN2A* gene are associated with FAMMM, although there has been wide variability in the reported prevalence of *CDKN2A* mutations in patients with FAMMM [49]. The risk of PC in kindreds with FAMMM is 13- to 22-fold greater than the average population [50,51].

Hereditary pancreatitis is associated with a markedly increased risk of PC, although it represents a very small fraction of PC cases [52,53]. It is an autosomal dominant disorder, most often associated with mutations in serine protease 1 gene (*PRSS1*) which encodes cationic trypsinogen, that accounts for a small fraction of cases of chronic pancreatitis [54]. Rebours et al. [53] evaluated 78 families including 200 patients and calculated a cumulative risk of PC in affected family members of 10, 19, and 54% at 50, 60, and 75 years, respectively. In this population, the risk was even higher in smokers and in patients with diabetes mellitus (DM). In fact the risk of PC within hereditary pancreatic kindreds varies upon their genotype. For example, an individual with true hereditary pancreatitis and the p.R122H mutation of *PRSS1* has been shown to have a >50 times increased risk of pancreatic neoplasia [55]. Conversely, an individual with CFTR associated familial pancreatitis has a less elevated risk [56].

Clinical risk factors

Clinical risk factors are relatively broad and non-specific, such as: age, smoking, DM, obesity, chronic pancreatitis and *Helicobacter pylori* infection. Some experimental data suggest a possible inhibitory effect of aspirin and other Nonsteroidal Anti-Inflammatory Drugs on pancreatic tumorigenesis, however epidemiologic studies in humans are conflicting [57–59]. Data regarding the impact of coffee and alcohol ingestion on the risk of PC have been conflicting [60–62]. In fact, considering the alcohol ingestion, a meta-analysis provides strong evidence for the absence of a role of

moderate drinking in pancreatic carcinogenesis, coupled to an increased risk for heavy alcohol drinking. Therefore, given the moderate increase in risk and the low prevalence of heavy drinkers in most populations, alcohol appears to be responsible only for a small fraction of all pancreatic cancers [63].

In terms of age, PC is rare before age 45, but the incidence rises sharply thereafter, with over 97% of cases occurring in this group of patients [64]. Exception should be made for the individuals with FPC or any form of hereditary pancreatitis, in whom PC can develop earlier.

Tobacco is the main recognized risk factor for PC and nowadays it contributes for a third of all PC cases in most areas of the world [65]. Tobacco carcinogens and metabolites may act in concert and have both genetic and epigenetic effects at early and later stages in pancreatic tumorigenesis [65]. Being a current smoker (compared with never smoking) significantly advanced the risk of developing PC by 7.6 years [66]. Although having quit for more than five years seems to reduce the risk to the same levels as nonsmokers [65].

The trends in PC mortality partly reflect patterns of tobacco consumption across generations, genders and localization. PC mortality began to drop in countries which first implemented strict tobacco control (including Northern Europe, the US, Canada and Australia), while mortality rates were still increasing in areas where reduction in use of tobacco products occurred later (i.e. Eastern Europe) [65].

Several epidemiologic studies described an association between DM and PC [67,68]. Diabetes was associated with an increased risk of PC (the summary RRs = 1.94; 95% CI, 1.66–2.27) [67]. Moreover, new-onset (<24 months) of DM may be an early indicator of PC. In fact, 85% of patients with PC have hyperglycemia and up to 60% DM, which is predominantly of recent onset. Late onset (>50 years of age) DM is associated with a 8 fold risk of developing PC in 3 years. Nevertheless, only approximately 1% of late onset DM subjects will develop PC [69].

The relationship between DM and PC is complex and it is not well established if the first is a consequence or a cause of pancreatic neoplastic change. DM secondary to an underlying disease of the exocrine pancreas is currently classified as type 3c DM by the American Diabetic Association. As mentioned, many patients with PC develop DM shortly prior to cancer diagnosis, which is often paradoxically ameliorated by surgical resection [70].

In combination with the increased risk for PC in those with new-onset DM, these data suggest that the tumor itself may induce DM. Supporting this hypothesis, some emerging data explore the concept of PC-induced paraneoplastic DM, including the identification of potential mediators of β -cell dysfunction, such as adrenomedullin [71]. If in the future, it will be possible to properly distinguish, in advance, type 2 DM from PC-induced type 3c DM, we will certainly have the key to identify high risk patients that should be screened for early PC detection.

Regarding anti-diabetic therapy, a meta-analysis of 11 studies, published by Singh et al. showed that sulfonylureas seem to be associated with increased risk of PC. However, the other anti-diabetic medications did not show protective or harmful association between its use and risk of PC in patients with DM [72].

Overweight or obesity during early adulthood is associated with a greater risk of PC and a younger age of disease onset [73]. The precise pathophysiological mechanisms linking obesity and PC are still under investigation, however, both systemic and local tissue effects and white adipose tissue inflammation appear to be a critical components [74].

Chronic pancreatitis patients have an estimated 2.2 fold risk for developing PC. It also shares several etiological factors with PC and represents its most challenging differential diagnosis. Biomarkers that can distinguish both entities may be suitable for PC early

detection. Targeting the natural history of chronic pancreatitis could be one step to prevent PC development [75].

A recent meta-analysis showed a weak but statistically significant association between *Helicobacter pylori* infection and PC. This relationship is prominent in Europe and East Asia, but not in North America [76].

Who should be screened?

Since the incidence of PC in the general population is low with a lifetime risk of 1.3%, population-based screening is not recommended [27].

Despite having an incomplete understanding of the risk factors for PC, certain groups have been identified as being at a higher risk based on family history or identifiable genetic predisposition.

As a matter of fact, the International Cancer of the Pancreas Screening (CAPS) Consortium proposed that PC screening should be considered in this group of patients, defined as having more than 5% of lifetime risk or fivefold increased relative risk for PC, fivefold increased relative risk for PC [27,77]. Nevertheless, recommendations are primarily based on evidence of increased risk, rather than a proven efficacy of screening.

Family history data remains the most important tool to quantify PC risk [77] and screening is actually recommended for individuals with: i) three or more blood relatives with PC, with at least one affected FDR; ii) at least two affected FDRs, and iii) two affected blood relatives with PC, with at least one FDR [77]. In fact, Klein et al. have shown that the risk in FPC kindreds depends on the number of relatives with PC; with the risk of PC increased in individuals with three (OR = 32.0; 95% CI, 10.2–74.7), two (OR = 6.4; CI, 1.8–16.4), or one (OR = 4.6; CI, 0.5–16.4) FDRs with PC [78].

Actually, there is no consensus on whether to screen individuals without an affected FDR, including individuals with a relative with young-onset PC, or patients with new-onset diabetes [27].

In relation to hereditary cancer syndromes, the same CAPS Consortium proposed for screening, i) all patients with PJS, regardless of family history of PC; ii) CDKN2A/p16 carriers (FAMMM) with one affected FDR; iii) BRAC2 mutations carriers with one affected FDR (or two affected non-FDR family members; iv) PALB2 mutation carriers with one affected FDR; v) Mismatch repair gene mutations carriers (Lynch syndrome) with one affected FDR.

More recently, the American College of Gastroenterology has published a Clinical Guideline about “Genetic testing and management of hereditary gastrointestinal cancer syndromes”, where some differences in relation to CAPS Consortium recommendations can be noted [79]. These guidelines advocate to screen PC in mutation carriers from hereditary syndromes associated with PC, namely PJS, hereditary pancreatitis and FAMMM, and members of FPC kindreds with one PC-affected FDR. Because of lower risk of development of PC in BRCA1, BRAC2, PALB2, ATM and Lynch Syndrome families, they state that screening should be limited to mutation carriers with a first or second degree relative affected with PC.

How to screen: clinical history, genetic testing, imaging studies and biomarkers

As mentioned before, it is crucial to access the risk of PC for each patient to ultimately stratify high risk groups. A detailed medical history is essential to identify some of the most important factors, including: PC's family history, smoking and drinking habits, presence of DM (mainly if it is a recent diagnosis), history of chronic pancreatitis and obesity. Physicians should pay attention particularly to patient's family history (number of affected family

members and the relationships among at-risk individuals), as it remains the main tool to quantify PC risk [26].

Gene testing can identify family's underlying genetic susceptibility, but it has a limited role as the genetic basis of most of the inherited susceptibility to PC remains unexplained [26].

Considering the imaging studies, there is no consensus regarding the best screening method to PC screening. The main modalities of imaging to detect a pancreatic tumor are: endoscopic ultrasound (EUS), multidetector computed tomography (MDCT), MRI, magnetic resonance cholangiopancreatography (MRCP), endoscopic retrograde cholangio-pancreatography (ERCP) and positron emission tomography (PET).

Abdominal ultrasound, besides its unequivocal advantages (easily available, inexpensive, safe), can not be recommended as a screening modality for PC screening [27]. In fact, its sensitivity is no more than 70% and, more importantly, it is a technique that frequently can not accurately evaluate the entire pancreatic gland [80].

MDCT is currently the abdominal imaging test of choice for pancreatic disease, particularly for diagnosis of solid tumors and staging of PC [81]. Despite its high accuracy for detecting and staging of pancreatic malignancies, the sensitivity of MDCT may be suboptimal, as MDCT misses some target lesions in PC screening scenario. The sensitivity of thin-section, triple-phase helical CT to detect lesions <2 cm is only 70%–80% [82]. Further, there is also a concern for ionizing radiation exposition, hence, CT is not the ideal screening or surveillance imaging test for high-risk individuals (HRIs).

MRI may be an appropriate choice for noninvasive screening of HRIs because it is able to image the entire abdomen and pelvis, avoiding radiation exposure. MRCP is able to image pancreatic ductal anatomy noninvasively (unlike ERCP) and small cystic lesions such as IPMNs. Preliminary data from high-risk patients who underwent surgical resection (CPAS3 study) suggest that MRI/MRCP may be superior to CT, particularly for detection of IPMNs (71% vs 14%, $p < 0.001$) [83].

Recently, MRI capabilities have been improved by the addition of diffusion-weighted imaging (DWI) which brought functional aspects into conventional morphologic evaluation. The high contrast resolution of PDACs on DWI techniques is useful for the identification of even very small lesions, therefore allowing earlier diagnosis. Furthermore, the intravoxel incoherent motion (IVIM) model is an advanced DWI technique that allows the study of molecular diffusion and blood microcirculation (perfusion). Although not routinely integrated into clinical practice, it represents a promising technique to characterize pancreatic solid lesions, specially on the differentiation between PDAC and mass forming pancreatitis [84].

However, nowadays EUS is a crucial tool being used in HRIs screening programs [85]. Although it is an operator-dependent endoscopic procedure, with inherent risks of invasiveness and sedation, eventually limiting its role in a widespread screening and surveillance program, EUS has gradually reached the widely accepted status of the most accurate tool for investigation of pancreatic diseases. It can provide high-resolution images of the pancreas without the risk of radiation exposure and can image mural nodules (focal thickening of the wall in branch duct IPMNs), which are associated with increased risk of malignancy. Analysis of HRIs enrolled in a screening program who underwent surgical resection suggests that EUS can detect almost twice as many neoplastic lesions as CT or MRI/MRCP [83]. Also, previous studies [86,87] using EUS as a screening tool for HRIs have reported detection of asymptomatic precancerous branch duct IPMNs, large PanINs, incidental pancreatic endocrine tumors and ductal adenocarcinomas. Recently, the possibility of complementing the EUS

examination with elastographic and contrast-enhanced ultrasound (CEUS) studies has opened a new and promising field of investigation, in order to better detect and characterize pancreatic focal lesions [86–89].

EUS-guided FNA, besides its usefulness in solid lesions, has also been used to investigate pancreatic cystic lesions and can provide a cytological diagnosis of IPMN and MCN in almost 70% of the cases [90].

Besides elastography and CEUS, confocal laser endomicroscopy has recently appeared as a technological improvement to EUS with a particular role in cystic lesions evaluation. This technique consists of a real time direct visualization of the epithelial lining at a microscopic level, theoretically allowing the identification of suspicious architectural changes. However, at this moment, its high cost, steep learning curve and low availability have limited its use besides investigational trials [91].

Currently, there is no biomarker with adequate sensitivity and specificity that can be used for routine clinical PC screening [92]. Given the typical late stage of disease at the time of diagnosis, there has been much effort invested in identifying accurate tumor markers to aid in earlier diagnosis of PC.

The current most widely used biomarker for PC is CA 19-9, and, to a lesser degree, carcinoembryonic antigen (CEA) and genetic markers such as KRAS and p53 [93].

CA 19-9 is a cell surface glycoprotein expressed by PC cells. However, it is not specific, as it can also be found in normal pancreatic and biliary duct cells and gastric, colonic, endometrial and salivary epithelia. Consequently, CA 19-9 is not routinely used for diagnosis because of the unacceptably high rate of false-positive results, with specificity ranging from 33% to 100% [94–96]. CA 19-9 is also associated with imperfect sensitivity, ranging from 41% to 86% [94,96].

Moreover, approximately 4%–15% of the general population do not express the Lewis antigen and therefore do not have detectable CA 19-9 levels [96–100]. In patients with resectable PC, only 65% exhibit an elevated level of CA 19-9. The marker is also inadequate to differentiate between PC and chronic pancreatitis, as up to 40% of the patients with chronic pancreatitis can exhibit elevated levels of CA 19-9 [97,101].

Given its performance characteristics as a biomarker in the general population, serum CA 19-9 is used primarily as a prognostic tool when managing patients with late stage disease, or in monitoring patients for recurrence [97,102].

Considering the genetic markers, Eshleman JR et al., taking into account the possibility that the identification of KRAS mutation in pancreatic fluid analysis might identify microscopic neoplasias in the pancreatic duct system, showed interesting results. Seventy-three percent of patients with PC and 50% of asymptomatic individuals with a high risk for PC had KRAS mutations detected in pancreatic juice. However, 19% of controls, without pancreatic abnormalities based on imaging analyses, had also this mutation that likely arises from small pancreatic intraepithelial neoplasia lesions [103].

MicroRNA (miRNA) are small non-coding RNA about 18–24 nucleotides in size. Evidence indicates that miRNAs regulate gene expression at the post-translational level in almost every biological event and play important roles in tumorigenesis, cancer development, migration and metastasis. Numerous investigations have evaluated the miRNA signature of PC, utilizing pancreatic tumor tissue and cell lines, searching for biomarkers and their association with tumorigenesis and patient survival [104,105]. However, tissue-based miRNA signature profiling is limited to the availability of tissue specimens. Thus, a simple, noninvasive procedure, such as blood based signature profiling, would be ideal for detecting PC at earlier stages.

Recent studies have shown that miRNAs are relatively stable and can be readily extracted and detected in bodily fluids such as blood plasma. Among others, miR-192 is very promising showing increased levels in serum of PC patients compared to healthy controls with sensitivity towards cancer at 76% and specificity at 55% [106]. Another potential biomarker is miR-18a, which occurs at high levels in tumor tissue as well as in plasma of cancer patients. In addition, miR-18a levels were found to be significantly reduced after tumor resection. Other studies are focused on using combination of several circulating miRNAs to discern various stages of PC from cancer-free controls and CP [107,108]. Recently, a study introducing a promising panel of circulating miRNAs for early blood-based diagnosis of PC has been presented [109]. This study, from Ganepola et al. [109], employed high throughput screening as a screening tool to identify blood-based circulating miRNA markers to detect early stage PC. Two levels of confirmation were performed to ensure the validity of the identified miRNA targets. First, a panel of potential miRNA markers was generated and confirmed using a more specific and sensitive secondary assay, real-time quantitative reverse transcription polymerase chain reaction. Second, the confirmed panel of miRNA markers (miR-642b, miR-885-5p, and miR-22) was independently validated with different experimental procedures and instruments, by independent personnel, and at a different institution, to diagnose a new cohort of patients and controls. miR-885-5p and miR-22-3p each demonstrated high sensitivity of 82% for cancer case identification and relatively high specificity of 73% and 82%, respectively, for identifying healthy controls. For miR-642b-3, a high sensitivity of 82% to identify cancer cases was demonstrated, but a lower specificity for identifying healthy controls of 55% was observed. When using the optimal cut-point, the combination of all three miRNAs yielded a sensitivity and specificity of 91%.

Ongoing research has also developed other areas of investigation: a recent article from Melo et al. [110] demonstrated that Glypican-1 identifies cancer exosomes and detects early PC. Exosomes are lipid-bilayer-enclosed extracellular vesicles that contain proteins and nucleic acids. They are secreted by all cells and circulate in the blood. Using mass spectrometry analyses, the authors identified a cell surface proteoglycan, glypican-1 (GPC1), specifically enriched on cancer-cell-derived exosomes. GPC1+ circulating exosomes (crExos) were monitored and isolated using flow cytometry from the serum of patients and mice with PC. GPC1+ crExos were detected in the serum of patients with cancer with absolute specificity and sensitivity, distinguishing healthy subjects and patients with a benign pancreatic disease from patients with early- and late-stage PC. Levels of GPC1+ crExos correlate with tumor burden and the survival of pre- and post-surgical patients. The importance of these findings is that GPC1+ crExos from patients and from mice with spontaneous pancreatic tumors carry specific KRAS mutations, and reliably detect pancreatic intraepithelial lesions in mice despite negative signals by magnetic resonance imaging. GPC1+ crExos may serve as a potential non-invasive diagnostic and screening tool to detect early stages of PC that will possibly allow surgical curative therapy.

When should screening be started?

There are no clear guidelines concerning which is the most adequate age to start screening in HRIs. However, expert recommendations considered to initiate PC screening at the mean age of PC and/or the youngest age of onset of PC in the family. The average age at diagnosis of patients with FPC is in the 6th decade of life [111].

Some authors recommend that screening should be considered at the age of 40–45 years or 10–15 years younger than the

youngest relative with PC. The International CAPS Consortium voted 50% to recommend starting screening at age 50 [27].

The American College of Gastroenterology in its clinical guidelines about genetic testing and management of hereditary gastrointestinal cancer syndromes, advocate that surveillance for PC should be with EUS and/or MRI of the pancreas annually starting at age 50 years, or 10 years younger than the earliest age of PC in the family [79].

In patients with PJS, screening is recommended at age 30 [30], and in PRSS1 mutation carriers with hereditary pancreatitis it should begin at age 40, also due to young age of onset of PC in these patients [112].

As smoking has been described as an independent risk factor for PC in families with FPC, all HRIs should be strongly counselled against smoking [113]. However, there is no consensus as to whether to recommend initiating screening at an earlier age for current smokers [27].

In individuals with chronic pancreatitis, in addition to smoking cessation, a low-fat diet should also be recommended [30] and no recommendations are available regarding PC screening.

Does screening really work? the caveats

The key question to address in relation to early detection is whether or not survival of afflicted patients is actually increased and if the population death rate from PC is decreased, following an engagement in a screening program. As with the controversies in breast and prostate cancer screening, there is the chance that, despite earlier diagnosis, natural history of the cancer in affected patients would not be altered (lead-time bias). This form of selection bias, occurs when the screened population appears to have longer survival than unscreened patients. Nonetheless, the screening has had no actual impact on the natural history of the disease process [114].

Another problem concerning screening programs is the length bias that occurs because of the tendency of screening programs to identify tumors with a longer natural history [5,115].

These issues of lead-time and length bias will have to be addressed in carefully designed and conducted trials once a screening strategy with high accuracy can be identified [5].

Moreover, as already mentioned, since the incidence of PC in the general population is low, with a life-time risk calculation of 1.3%, screening is not recommended for the general population. Therefore, only individuals considered to be at high risk of developing PC are selected for PC screening. Taking into account that only 10% of patients with syndromic risk factors or a family history are diagnosed with PC, a significant amount of PC cases in general population would be missed [5].

Another problem related with the PC screening program is the absence of a serologic biomarker of early disease or even for a precursor lesion with potential to progress towards PC. Until now, CA 19-9 is the best PC tumor marker, however its sensitivity for resectable cancer is nearly 25% and even lower for stage I PC [116].

Screening studies performed have used different imaging techniques, such as: EUS, ERCP, and cross sectional imaging (CT and/or MRI with MRCP). Considering all the studies performed, in a total of 410 patients with high risk of PC, 10% of them underwent surgical resection due to malignancy suspicious. Eight cases of invasive PDAC were detected resulting in diagnostic yield for malignancy of 1.95% (8/410). In 9% of the patients benign lesions (IPMN or PanINs) with malignant potential were identified and several unrelated lesions without risk of progression to cancer, such as serous cystadenoma and pancreatitis were identified in numerous patients [5]. In conclusion, these studies showed that an important limitation of imaging-based screening programs is the relatively

high rate of identification of harmless lesions that subsequently require additional invasive evaluation [5].

These results highlight the risk of overtreatment using available screening tests. The risk of overtreatment for pancreatic screening is magnified by the risks of morbidity and mortality (3.8%) of pancreatic surgery [117].

Despite the aforementioned issues related to screening, a promising strategy has been reported in Japan. A regional network between Specialists in PC and General Practitioners has shown an important role for early PC diagnosis. The first project was implemented in the rural city of Onomichi, with approximately 150,000 habitants, where a total of 6475 cases was evaluated, with several dedicated imaging methods (CT, MRI, EUS and ERCP). Considering the screened patients, a total of 399 (6.2%) was histologically diagnosed as PC, 16 of them representing in situ neoplasia. The spread of this concept to other rural areas of Japan will reinforce the regional network for early diagnosis of PC [118].

Detection of pre-invasive lesions (PanIN, IPMN and MCN): the hidden key

Recently, there is a growing enthusiasm on the identification of highly specific biomarkers for PC. Ideally, these biomarkers should be capable of identifying the neoplastic process in its pre-invasive stage, which means diagnosing the precursor lesions of PC or the PC itself at a very early and curable stage.

Three noninvasive precursor lesions of PC have been described: i) PanIN, ii) IPMN and iii) MCN [119].

IPMN accounts for less than 10% of all pancreatic neoplasms and particularly IPMNs with high-grade dysplasia are associated with a significantly increased risk of invasive PC [120,121]. In fact, the detection and posterior treatment of IPMNs with high-grade dysplasia should be considered a success of a screening/surveillance program according to the International CAPS Consortium [27]. However, the frequency and rate at which IPMNs progress to invasive PC are still unknown.

Genetic changes underlying IPMN carcinogenesis are progressively been described and GNAS mutations seems to play an important driving role in this IPMN-specific pathway [122]. Shi et al. [121] showed that high-grade incipient IPMNs were only observed in patients with a strong family history comparing to others with sporadic disease.

IPMN is characterized by papillary growths within the pancreatic ductal system with thick mucin secretion [120]. Based on imaging studies and/or the histology and taking into consideration the site and extent of involvement, IPMNs can be classified into three types: MD-IPMN (main duct intraductal papillary mucinous neoplasm), BD-IPMN (branch duct intraductal papillary mucinous neoplasm), and mixed type. IPMNs are also classified into four histopathological types based on the cytoarchitectural features and immunophenotypes: intestinal (18–36%), gastric (49–63%), pancreaticobiliary (7–18%), and oncocytic (1–8%) types [120,123]. The intestinal type is the most frequent one among MD-IPMN and carries the higher risk of invasive carcinoma, more commonly of colloid type. On the contrary, the gastric type is the most common subtype among BD-IPMN and it is usually characterized by a low proliferative activity with a very low risk of malignant transformation. The pancreaticobiliary type, recognized as the high-grade version of gastric type, is associated with at least high-grade dysplasia, and when invasive carcinoma is present is frequently of the tubular type. The oncocytic type proliferation is associated with atypical cytology with high-grade dysplasia [124].

MD-IPMN is characterized by segmental or diffuse dilation of the main pancreatic duct of >5 mm without other causes of obstruction. The incidence of malignancy in MD-IPMN vary from

57% to 92% and from 6% to 46% in BD-IPMN [120].

In order to diagnose and evaluate IPMNs, MRCP should be considered as the initial imaging technique as it was shown to be more reliable comparing with CT, with clear advantages in evaluating the relation of the cystic lesion(s) with the pancreatic ductal system [125]. EUS adds a further dimension in the diagnosis of IPMN by improving the accuracy of the assessment of pancreatic parenchyma [126] and allowing the possibility to perform biopsy that can be useful to classify preoperatively IPMN according to its histopathological subtypes [127].

Takanami et al. [128] showed that fludeoxyglucose (18F)-PET/CT scan was found to be superior to contrast-enhanced CT providing additional information for the differentiation between benign and malignant IPMNs of the pancreas with mural nodules [128].

Despite of all the advances in radiological investigations to facilitate the diagnosis of IPMN, the concern remains in differentiating a benign IPMN from a malignant lesion.

Another important fact is the association between IPMN and synchronous and/or metachronous PDAC. In 1997, this fact was first reported by Tanaka et al. [129] who described a carcinoma in situ concomitant with a small benign BD-IPMN. Since then, this association has been repeatedly described in several studies [130]. This phenomenon highlights the chance of early PDAC detection during IPMN follow-up with CT, MRI and EUS.

Before the end of the last century, MCN and IPMNs were frequently confused. In fact, it was only after 1996, when the World Health Organization distinguished between IPMNs and MCNs, emphasizing the presence of ovarian stroma in the latter, that these two entities began to be considered as separate ones. Nowadays, they represent two distinct neoplasms with different biologic behavior, pathologic features, and prognosis [131].

MCNs are defined as mucin-producing and septated cyst-forming epithelial neoplasia of the pancreas with a distinctive ovarian-type stroma. Usually solitary, their size ranges between 5 and 35 cm. They usually have a fibrotic thick wall and do not communicate with the ductal system [31]. MCNs are rare and, in most series, less common than IPMNs and serous cystic neoplasms, with a female to male ratio of 20:1 with a mean age at diagnosis between 40 and 50 years [132]. While predominantly located in the body and tail of the pancreas [133,134] its localization in the head is associated with a more prevalent malignant transformation (mucinous cystadenocarcinoma) [135].

The increasing degree of dysplasia and tendency for invasion have been correlated with activating point mutations in the KRAS gene and mutations in the TP53 gene [136,137]; the discovery that the inactivation of SMAD4/DPC4 in the epithelium of the invasive MCNs, but not in the ovarian-like stroma, could suggest that the ovarian-type stroma is not neoplastic [138]. Invasive carcinoma incidence in MCN varies between 6% and 36% [31,135,139]. Preoperative diagnosis depends on a combination of parameters, including clinical features, tumor markers, CT, MRI, EUS and PET. EUS, once again, has a major role in the diagnosis of these lesions, as it allows better evaluation of the wall as it may show separation or nodules within the cyst. Furthermore, EUS can be used to obtain aspiration of the cyst contents and to perform a biopsy of the wall.

PanIN lesions are composed of columnar to cuboidal cells with varying amounts of mucin and different degrees of cytological and architectural atypia [140]. They are classified into 3 grades and are characteristically asymptomatic. PanIN-1A (flat) and PanIN-1B (papillary) are low-grade lesions with minimal cytological and architectural atypia. High-grade PanINs (PanIN-3) are characterized by severe cytological and architectural atypia and are also described as “carcinoma in situ” [140].

The prevalence of PanIN lesions is 55% in cadavers of patients that were over 80 years old and they are also more prevalent in FPC

kindreds and in patients with PC [121,141,142]. Similar to IPMNs, PanINs lesions produce obstructive lobular atrophy, which is likely the source of the chronic pancreatitis-like changes seen in these patients [143].

PanINs are considered precursor lesions in the stepwise progression from intraepithelial to invasive pancreatic neoplasia. This morphological progression is paralleled by an accumulation of genetic changes in which activating KRAS mutations are thought to be the driving force.

Unfortunately, PanINs are not yet detectable by cross-sectional imaging (MRI or CT) or EUS. However, as previously described, multifocal PanINs can produce multifocal lobulocentric atrophy giving to the pancreas an appearance similar to that of chronic pancreatitis that can be possibly detected by EUS.

Nonetheless, even using several techniques, it can be remarkably difficult to conclusively differentiate a chronic pancreatitis from a malignant mass. Moreover, PanIN can be a concomitant finding with a PDAC.

In the future, molecular markers in the pancreatic juice or other patient's fluids may help to solve this dilemma [140].

Management of pre-invasive lesions

Theoretically, the early detection and surgical treatment of pancreatic precursor lesions can prevent the development of a lethal cancer [119,144]. This simple principle emphasizes the importance of early recognition of these lesions in patients who are at high risk for PC, allowing early therapeutic intervention, either surgically or with soon-to-be-developed targeted therapies.

Despite the importance of PC prevention throughout surgical treatment of precursor lesions, it is crucial to consider the risk of overtreatment, mainly in patients who are not at high risk for PC. Therefore it is imperative to have international recommendations for the management of IPMN and MCN, so far the lesions that can be identified by cross-sectional imaging and/or EUS.

Nevertheless, whether patients with pancreatic cysts have a higher long-term risk of PC has still not been well established. Recently, Munigala et al. [145] published a retrospective study of Veterans Administration, where patients noted to have pancreatic cysts on CT/MRI were followed from 1998 to 2007. During the study period, the incidence rate of PC among patients with pancreatic cysts was 5.08 against 0.32 per 1000 patient-years in the control group. The hazard ratio of PC in all patients with cysts was 19.64 (95% CI, 12.12–31.82; $p < 0.0001$) when compared with the rest of the patients without cysts.

There has been a continuous effort of International Societies to establish guidelines for the management of patients with pancreatic cysts [146]. The American Society for Gastrointestinal Endoscopy, in 2005 established preliminary practice recommendations for EUS-FNA and risk analysis, but the role of cyst fluid tumor markers was not well defined [147]. In 2006, the International Association of Pancreatology published a set of guidelines (Sendai) [148], where a management algorithm was developed establishing intervention based on the size of the cyst and the presence of high-risk stigmata (mural nodules, dilated main duct or positive cytologic results). In 2007, the American College of Gastroenterology [149] established a consensus to perform EUS-FNA in cysts >2 cm to differentiate between mucinous and nonmucinous ones. For this purpose, carcinoembryonic antigen level in cyst fluid was considered the best marker.

In 2012, the Sendai guidelines were updated in a meeting of the International Association of Pancreatology, that took place in Fukuoka [125]. Patients with a “worrisome feature”, namely: cyst >3 cm, thickened/enhanced walls, main duct 5–9 mm, non-enhancing mural nodule, or abrupt change in caliber of pancreatic

duct or distal pancreatic atrophy were elicited to EUS-FNA. Patients without “worrisome features,” were managed based on the size of the cyst: cysts <1 cm were enrolled into a CT/MRI surveillance in 2–3 years; cysts 1–2 cm were placed into a yearly surveillance program; larger (>2 cm) cysts were managed with EUS-FNA, and surgery being recommended for cysts >3 cm in a well fitted patient. If any “high-risk stigmata” was present (nodule, duct dilation, or jaundice) on EUS, surgical resection was recommended.

These guidelines were validated through some retrospective studies [150–152], that showed a superior accuracy for the Fukuoka guidelines when compared to the Sendai ones.

The American Gastroenterology Association (AGA) commissioned an evidence-based review of the diagnosis and management of pancreatic cysts [153], more conservative than Fukuoka guidelines, particularly in the threshold for the use of EUS-FNA. These guidelines have been questioned by some authors, and a relevant recent publication by Singhi et al. [154] concluded that they were inaccurate in detecting pancreatic cysts with advanced neoplasia in a clinicopathologic study of 225 patients with supporting molecular data. This, in part, can be attributed to the fact that only studies that reported the presence or absence of invasive malignancy were evaluated in AGA analysis. The diagnosis of carcinoma in situ or high-grade dysplasia was not considered to be an invasive malignancy in these settings. In addition, because the AGA guidelines manage all neoplastic pancreatic cysts similarly, patients with serous cystadenoma will continue to perform costly and unnecessary MRI surveillance.

In summary, many recommendations are now available to guide management of patients with pancreatic cysts, being difficult to judge which one should be adopted. The authors agree with William Brugge [146] when he states that the most important is to decide whether the guidelines should be designed to detect early neoplasia (eg, high grade dysplasia), as in Sendai-Fukuoka ones, or late (invasive) neoplasia, as in the case of AGA recommendations.

Having in mind that “worrisome features” or early neoplasia in the elderly can be managed conservatively and that decision to risk a pancreatic resection must take into account also the patient's global health and the site of the cystic lesion (the threshold for resection a lesion in the tail should be lower than for head ones) [155], our personal daily practice follows more the principles stated in Sendai-Fukuoka guidelines. We are then in line with the International CAPS Consortium [27] that recommends to follow in most cases the Fukuoka Guidelines. We also believe that if we want to change the dark picture of PC, early neoplasia should be resected in the young, whereas finding advanced lesions will certainly have no impact on patient outcomes.

Taking all this in account, we can generally admit that resection is recommended in all surgically fit patients with MD-IPMN. If the margin is positive for high-grade dysplasia, additional resection should be attempted to obtain at least moderate-grade dysplasia [27].

In relation to BD-IPMN, indications for resection are more conservative and should take into consideration the presence of “worrisome features” as well as “high-risk stigmata” as previously mentioned. A BD-IPMN of >3 cm without “high-risk stigmata” can be observed without immediate resection [156,157].

The natural history of MCN is still unknown and non-operative management would require years of follow-up based on high-resolution imaging associated with high costs [125,139]. Therefore, surgical resection is recommended for all surgically fit patients with MCN, given the relatively young age of most patients and the risk of progression to invasive MCN. For MCNs of <4 cm without mural nodules, laparoscopic resection as well as parenchyma-sparing resections and distal pancreatectomy with spleen

preservation should be considered [125,158].

Small (<1 cm diameter) lesions identified as solid by EUS are difficult to manage, particularly when not detected by MRI or CT [159]. These lesions can be aspirated by EUS-FNA, but the yield for these lesions is low. Some indeterminate solid lesions identified only by EUS are cancers, but they can be benign lesions, such as non-metastatic pancreatic neuroendocrine tumors [160] or low-grade PanIN with focal associated lobulocentric parenchymal atrophy [161]. Although there is no strong consensus on the need for additional tests, it is advisable for indeterminate solid lesion and/or indeterminate main duct stricture, to repeat imaging at 3 months, if surgery is not imminent or possible [29].

The future

Recent advances in molecular biology during the last two decades have allowed a better understanding of the importance of multistep pancreatic carcinogenesis and its relevance in clinical practice. The understanding of PC precursor lesions natural history is a matter of great importance. The critical role of PanIN lesions has become more clear. Advances in proteomic analysis of these lesions may allow us to reliably and accurately target early lesions for chemoprevention and more advanced lesions for early resection [30].

Also, all the progresses that occurred in the last decades in relation to diagnostic techniques and therapeutic management of PC improve the scenario of PC screening, making it more feasible. Numerous studies have demonstrated that current imaging modalities (EUS and MRI/MRCP) have been improving its accuracy in detecting PC precursor lesions, with significant yield in appropriately selected, HRIs. However, the optimal approach and frequency of screening has yet to be established.

Despite of all these advances, prognosis remains dismal with an overall five-year survival of less than 5% [162], being the lowest-year survival rate of all cancers [163].

This gloomy reality may be explained, at least in part, by the fact that the more expressive improvements in PC early detection have been made in HRIs populations, namely FPC kindreds and individuals with hereditary cancer syndromes, which represent 10% of the spectrum of PC. So, a lot of efforts should be dedicated regarding sporadic PC.

Concerning this, a big step was done by the Kenner Family Research Fund that conducted the inaugural Early Detection of Sporadic Pancreatic Cancer Summit Conference in 2014, in conjunction with the 45th Anniversary Meeting of the American Pancreatic Association and Japan Pancreas Society. It was clear from the summit deliberations [164] that only strategically designed collaboration among investigators, institutions, and funders will lead to significant progress in early detection of sporadic PC, in order to improve overall PC patients survival.

First of all, collaborative studies are needed in order to understand the natural history of precursor lesions. Relating to risk factors for PC susceptibility, genes may be discovered in the near future that should improve our ability to identify individuals who would benefit most from pancreatic screening.

Finally, it is crucial to identify biomarkers that will be able to recognize precursor lesions in apparently normal individuals. Moreover, application of these biomarkers to imaging studies would be another important key allow early detection of these precursor lesions, in order to treat them adequately and in time, and definitely modify the dismal scenario of PC worldwide.

Hopefully, in brief, PC patients will be able to look ahead and think, like the baseball legend Yogi Berra, that “The future ain’t what it used to be”.

Author contributions

Pedro Moutinho-Ribeiro and Rosa Coelho: Design, conception and paper writing.

Marc Giovannini and Guilherme Macedo: critical revision and final approval.

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3.3.2.

PAPER 2

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Review Article

Early pancreatic cancer — The role of endoscopic ultrasound with or without tissue acquisition in diagnosis and staging

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ABSTRACT

Pancreatic cancer (PC) is one of the deadliest cancers with a 5-year overall survival of less than 6%. Due to its insidious clinical course and unspecific symptoms, the diagnosis is usually late, with only 15–20% patients presenting with potentially curable disease. It is, therefore, extremely important to identify patients with PC at early stages of the disease when tumors may be amenable to surgical resection. For unresectable and borderline resectable PC it is consensual to perform a biopsy to have a cyto/histological confirmation of malignancy before treatment. However, for patients presenting with promptly resectable disease, the role of biopsy is more debatable. There are, in the literature, arguments both for and against the usefulness of a preoperative biopsy. Endoscopic ultrasound (EUS) is an important technique assisting in the diagnosis and staging of PC. EUS-guided tissue acquisition is a well-established tool to demonstrate the malignant nature of a pancreatic lesion. This review focuses on the role of EUS in the diagnosis and staging of PC, and highlights the controversy related to the role of EUS-guided tissue acquisition in the preoperative assessment of patients presenting with promptly resectable tumors (early PC).

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1. Introduction

Pancreatic cancer (PC) is a fatal disease with a 5-year overall survival of less than 6% [1,2]. A recent European study showed that PC is the fourth leading cause of cancer-related death in men and women [3,4]. Over the last 20 years, prognosis of PC has not improved, with 49,000 new diagnoses and 41,000 deaths in the United States in 2015 and a rising number of deaths in Europe, from 75,439 in 2009 to 82,300 in 2014 [2,4]. It is estimated that PC will become the second leading cause of cancer-related death in the United States during the next decade [5].

The dismal prognosis of PC mostly stems from the late stage at which PC is usually diagnosed. Indeed, only 15–20% of patients present with potentially resectable tumors [4,6]. Cancer-related symptoms, such as weight loss, abdominal pain, nausea and vomiting, changes in bowel habits and jaundice, occur only at an advanced stage. New-onset diabetes is common in patients with PC, usually manifesting during preclinical or early stages of PC.

Although still controversial, it is possible that it may serve as clue for the early detection of PC [1,4].

There is no single definition of early PC. It may be defined based on resectability, size or curability. A restricted definition is related to the concept of maximum curability of the disease, defining it as minute (≤ 10 mm) well differentiated stage I lesions. A broader definition that is generally accepted considers a small (< 20 mm) localized lesion, promptly eligible for surgical resection [7]. Resectable PC is defined when there is no distant metastasis and no evidence of superior mesenteric vein or portal vein distortion or absence of clear fat planes around celiac axis, hepatic artery and superior mesenteric artery. On the other hand, borderline resectable and locally advanced PC is defined by the presence of variable degree of invasion of surrounding structures, such as superior mesenteric vein, portal vein, confluence, duodenal wall, gastric wall and/or biliary system. Patients presenting with this group of lesions are nowadays candidates to neoadjuvant chemotherapy with or without radiotherapy with the aim of downstaging for future resectability [8,9]. Lastly, non-resectable and/or metastatic disease are by far the most common presentations. In these last scenarios a definitive cyto/histological diagnosis is mandatory before oncological treatment is started. However, there is controversy in case of patients with promptly resectable PC. The question is whether a pre-surgical biopsy is always necessary.

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Over the last years, EUS has emerged as an important diagnostic tool for the evaluation of various digestive malignancies, including PC [4,10]. Importantly, it also allows the acquisition of tissue for pathological purposes, what has increased its role in the management of PC. EUS and EUS-guided tissue acquisition have shown to increase the diagnostic yield and staging accuracy of PC [4,10,11]. The role of EUS is also largely discussed in the PC screening scenario, taking into account its ability for detecting very small pancreatic lesions and its importance in the diagnosis and management of pre-malignant lesions, such as intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) [12].

Here, we review the literature to discuss the most recent developments in the preoperative management of patients with an early resectable PC. In particular, we discuss the contribution of EUS for an accurate pre-treatment diagnosis and staging.

2. Early diagnosis of pancreatic cancer: a field for EUS?

As mentioned above, the silent nature of PC contributes to its poor outcome. Yachida et al. estimated that the majority of patients with PC are only diagnosed in the last 2 of the 10-years tumorigenic process [13]. The earlier the PC is diagnosed, the better the prognosis will be [14]. Unfortunately, early detection of PC is generally difficult, since there are certain limitations for optimal diagnosis. A big effort in searching for screening strategies of PC is on-going over the last years. This approach has been considered an option to detect early stages and pre-cancerous conditions. This strategy has been recommended to individuals with more than 5% of lifetime risk for the disease [15]. However, it is well known that the incidence of PC is rather low (lifetime risk 1.3%), so general screening cannot be considered to be feasible [15]. Experts recommend to screen individuals with more than 10-fold increased risk, as the case of familial PC and several genetic syndromes associated with an increased risk (Peutz-Jeghers syndrome, familial atypical multiple mole melanoma, familial adenomatous polyposis, hereditary pancreatitis, Li-Fraumeni syndrome and Lynch syndrome) [16–19]. Recent meta-analysis has shown the potential role of magnetic resonance imaging (MRI) and EUS in the surveillance of high-risk patients for PC, for detecting pancreatic lesions, with similar accuracy for both methods [20,21].

Radiological studies are essential in diagnosis and staging of PC. However, they have important limitations, including the variability of the descriptive terminology to define disease extent and incomplete documentation of disease sites, which can affect the clinical decision-making process [22]. Multidetector computed tomography (MDCT), performed with a pancreatic protocol is usually considered as the method of choice for initial evaluation in patients with the suspicion of PC [22,23]. MRI has similar sensitivity and specificity; however, due to lack of wide availability, needed expertise, higher cost, and limited spatial resolution as compared to CT, it is not widely used as the primary imaging modality [4,22,24]. The main role of MRI is to evaluate isoattenuated pancreatic lesions and to better characterize indeterminate liver lesions identified at prior CT. Furthermore, it allows an exceptional view of the biliary and pancreatic duct, demonstrating the “double duct sign”, highly specific for PC [4,22,24,25].

The main imaging modality for the evaluation of pancreatic diseases, including solid pancreatic lesions, is EUS, with reported sensitivities between 87 and 100% [26–28]. EUS has shown a significantly better accuracy for the diagnosis of PC when compared to CT (sensitivity of 100% for EUS vs 86% for MDCT) [11,27,29–32]. EUS is particularly useful for identification of tumors <30 mm. In this setting, it presents a sensitivity of 93% compared to 53% for MDCT and 67% for MRI [27,30,33]. Despite this high accuracy, EUS has some limitations in the differential

diagnosis between PC and some non-neoplastic lesions, such as mass-forming chronic pancreatitis or autoimmune pancreatitis [26]. There are some strategies to improve this accuracy and overcome its limitations. Advanced imaging related to EUS has shown to improve the characterization of solid pancreatic lesions, among them contrast-enhanced EUS (C-EUS) and EUS-guided elastography. C-EUS is based on the administration of a specific contrast agent, which highlights the microvasculature of pancreatic masses during real-time evaluation. PC usually presents a hypovascular pattern, neuroendocrine tumors a hypervascular pattern and mass-forming chronic pancreatitis an isovascular one [34]. Yamashita et al. reported that hypoenhancement behaviour diagnosed pancreatic carcinoma with a sensitivity of 95% and specificity of 89%, findings supported by other authors [26,35,36]. EUS-guided elastography, a method that evaluates tissues' stiffness by measuring the degree of strain generated by external pressure, has demonstrated to be an excellent tool for management of pancreatic lesions [37]. Both a qualitative evaluation (based on the color map and pattern) and a quantitative evaluation (based on strain ratio or strain histogram) are available nowadays. Overall, hard lesions, shown by a blue pattern, high strain ratio (>10) or low strain histogram (<50), are mostly related to malignancy [38]. Although sensitivity of EUS-guided elastography for determine malignancy is very high it still lacks specificity [39,40].

Besides capable of improving lesion characterization, both C-EUS and elastography, also aid in the selection of the best area to perform EUS-guided tissue acquisition if indicated. Another strategy to improve diagnostic accuracy is the use of rapid on-site evaluation (ROSE) of the specimen by an experienced pathologist during the EUS exam. In fact, ROSE was found to result in fewer repeated procedures and higher percentage of definitive diagnosis as it determines whether the aspirate obtained is sufficient or not to permit a definitive diagnosis [41].

3. EUS in pancreatic cancer staging

The decision regarding resectability status should be made by consensus at multidisciplinary discussions, in high volume centers [4,8]. Although, PC staging was in the past based on the TNM classification, it is now defined with a more specific classification. PC should now be described as resectable, borderline resectable, locally advanced, unresectable or metastatic. All these features will guide treatment selection and constitute major prognostic factors for survival [4,22,42].

Both National Comprehensive Cancer Network (NCCN) and European Society for Medical Oncology (ESMO) guidelines consider MDCT angiography as a major modality [4,8]. Positron emission tomography does not add much information for staging PC and is not currently recommended. ESMO work-up suggests performing EUS for further assessment if initial MDCT does not reveal distant metastasis, whereas NCCN guidelines suggest that EUS is complementary to CT in selected cases [4,8,43]. In fact, it is generally accepted that EUS has an important role in preoperative staging. Besides its capacity to delineate tumor location and size, it presents a good accuracy for detecting lymph node metastasis, vascular invasion and predicting resectability. In a recent meta-analysis of 29 studies (including 1330 patients), EUS showed a pooled sensitivity of 69% and specificity of 81% for metastatic lymph node detection, and a pooled sensitivity of 85% and specificity of 91% for vascular invasion [10]. This analysis compared EUS and CT to detect arterial and venous invasion and concluded that both perform similarly for venous invasion but CT may be superior in assessing arterial invasion, namely the celiac axis and superior mesenteric artery [10]. There were no differences between EUS and CT in predicting resectability, with the former showing a pooled sensitivity

Table 1
EUS-guided tissue acquisition in diagnosis and staging of early pancreatic cancer.

<i>Arguments against a preoperative biopsy</i>
• Available imaging techniques have high diagnostic sensitivity.
• A negative result may not exclude malignancy.
• Potential seeding can hamper curative surgery.
• Can delay patient's treatment.
<i>Arguments favoring a preoperative biopsy</i>
• Has very good accuracy.
• Is a safe procedure.
• Increases surgeon's confidence.
• Increases patient's conviction.
• Avoids unnecessary surgery (in "mimickers").
• Allows the creation of a biobank for research purposes.

of 90% and specificity of 86%. Moreover, the possibility of performing tissue acquisition during EUS was also highlighted in this meta-analysis, especially its role in the characterization of atypical portocaval lymph nodes, ascitic fluid, peritoneal nodules and/or suspected liver metastasis. Thus, EUS may now be considered standard of care along with MDCT in preoperative staging of pancreatic carcinoma [10].

4. EUS-guided tissue acquisition in the diagnosis and staging of early PC: dismissible or recommended?

EUS is not only important to characterize morphologically the lesions, but also has the ability to obtain tissue samples [4,44,45]. EUS-guided tissue acquisition was first described in 1992 and is the preferred method for sampling pancreatic lesions [27,46]. EUS-guided tissue acquisition has a reported sensitivity of 79–98%, specificity of 71–100%, positive predictive value (PPV) of 96–100%, negative predictive value (NPV) of 33–85% and overall accuracy of 82–98%, with false negative and false positive rates of 12–14% and 0–5% respectively [45,47–52].

There is complete consensus regarding the need for tissue acquisition for a definite cancer diagnosis if administration of oncological therapy is indicated, as in the case of borderline resectable, locally advanced or metastatic disease [4,8]. However, controversy exists in the scenario of promptly resectable lesions: is a presurgical biopsy necessary or can these patients be proposed for immediate surgery without a prior PC definitive diagnosis? (Table 1).

According to the NCCN and the ESMO guidelines on PC patient's management, a biopsy proof of malignancy is not required for early resectable PC [4,8].

In this "early PC", the main objectives of EUS-guided tissue acquisition would be to accurately identify malignant lesions suitable for surgical resection and which type of resection would be needed (not all solid pancreatic lesion deserves the same surgical approach), but also to evaluate the need of neoadjuvant therapy, with recent data suggesting the role of this approach even in resectable pancreatic lesions. Moreover, patients with benign mimicking conditions can be spared from unnecessary surgery. The arguments more frequently pointed out for rejecting a preoperative biopsy are the high diagnostic sensitivity of the various imaging techniques, the fact that a negative result may not exclude malignancy and the possibility of hampering curative surgery because of potential seeding risk.

4.1. Arguments against a preoperative biopsy

As previously stated, imaging studies have a high diagnostic sensitivity for the diagnosis of pancreatic masses. MDCT has shown a sensitivity that can reach 90% and specificity as high as 99% [14,53,54]. Nonetheless, MDCT scan and EUS alone do not always allow a precise diagnosis, especially for tumors <2 cm or in the

setting of chronic pancreatitis [55,56]. In these circumstances, a EUS-tissue acquisition should always be considered.

When analyzing the results of EUS-guided tissue acquisition, and despite its excellent accuracy, the NPV still remains limited (approximately 60%). Moreover, chronic pancreatitis decreases diagnostic accuracy of tissue acquisition techniques, with difficult cytological interpretation. EUS-guided tissue acquisition can also have false positive results, usually in cases of chronic pancreatitis [27,57,58]. In patients with atypical/suspicious cytology but without a definitive diagnosis, it is recommended to repeat the puncture or treat as if the result was positive because surgery should never be delayed [59]. This is also related with another drawback of EUS-guided tissue acquisition is its ability in obtaining adequate cellular material, with reported unsatisfactory aspirates in 1.5–13% of cases [59,60].

Another issue against preoperative biopsy is its potential risk of tumor seeding [61]. There are anecdotal cases of tumor seeding after EUS-guided tissue acquisition along the needle tract, but with much lower frequency than with the percutaneous approach [62–64]. These cases were reported in tumors located on pancreatic body or tail, as in these locations tissue is obtained via transgastric approach that it will not be included in the surgical resection. On the other hand, lesions located on the head of the pancreas, which are sampled through the duodenum, should not harbor this risk, as the needle track will be included in the surgical specimen [65]. In 2013, Ngamruengphong et al. discussed this issue in a paper where 256 patients with similar baseline characteristics were analyzed: 48 patients in the non-EUS-guided tissue acquisition group and 208 in the EUS-guided tissue acquisition group. Recurrence data was available for 207 patients with a median length of follow-up of 23 months. Three patients had recurrence in the gastric wall: one (2.6%) in the non-EUS-guided tissue acquisition group vs. two (1.2%) in EUS-guided tissue acquisition group ($p = 0.46$). A total of 16 patients had peritoneal recurrence: 5 (12.8%) in the non-EUS-guided tissue acquisition group and 11 (6.5%) in the EUS-guided tissue acquisition one ($p = 0.19$) [66]. In multivariate analysis, undergoing EUS-guided tissue acquisition was not associated with an increased cancer recurrence or decreased overall survival, suggesting that it is not associated with an increased risk of needle track seeding. More recently, the same author, using the Linked Surveillance, Epidemiology, and End Results-Medicare data, identified a total of 2034 patients with locoregional PC who underwent curative intent surgery from 1998 to 2009 [67]. Patients who received EUS-guided tissue acquisition within the peridiagnostic period were included in the EUS-guided tissue acquisition group ($n = 498$) whereas patients who did not receive EUS evaluation or who underwent EUS without tissue acquisition were included in the non-EUS-guided tissue acquisition group. Overall survival and PC-specific survival were compared after controlling for relevant covariates. In multivariate analysis, EUS-guided tissue acquisition was marginally associated with improved overall survival (HR 0.84, 95% CI 0.72–0.99), but did not affect cancer-specific survival (HR 0.87, 95% CI 0.74–1.03). The authors concluded that preoperative EUS-guided tissue acquisition was not associated with an increased risk of mortality and suggested that it can be safely performed for the work-up of suspicious pancreatic lesions.

The argument that a preoperative biopsy can delay the treatment of these ideal surgical candidates should not be considered as most of these individuals should be evaluated and treated in tertiary well-experimented centers, where waiting lists for EUS-guided tissue acquisition procedures should not exist.

4.2. Arguments favoring a preoperative biopsy

EUS has demonstrated to be highly accurate in the diagnosis of solid pancreatic masses, being accepted as a minimally inva-

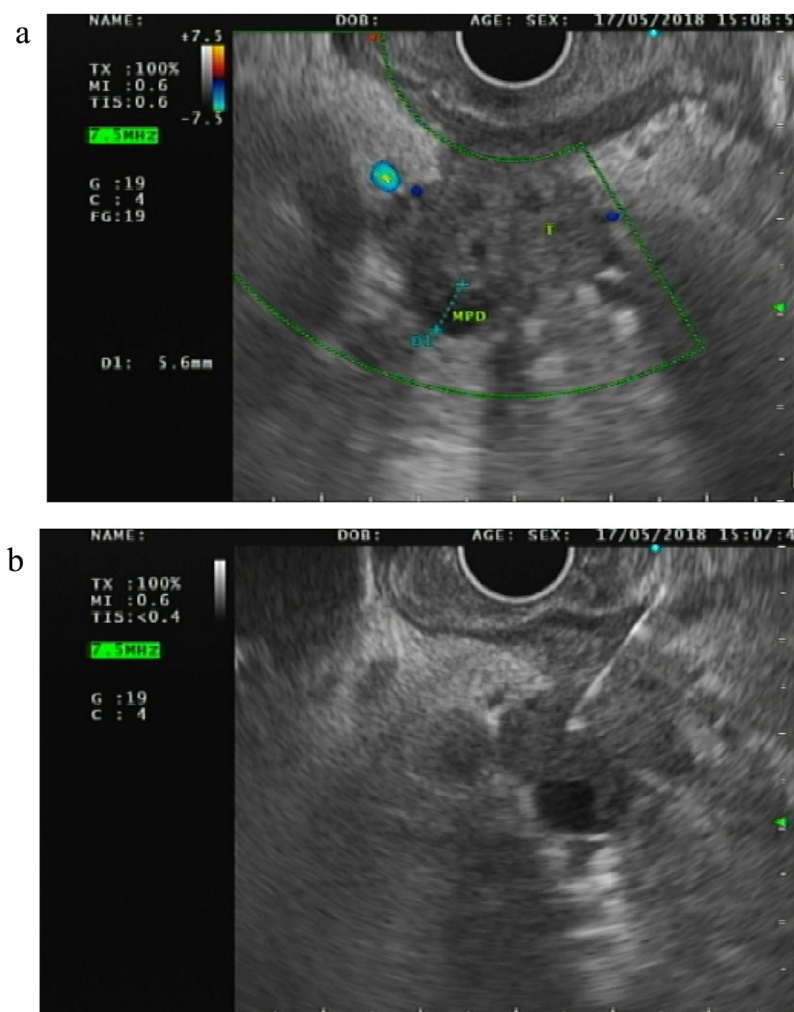


Fig. 1. (a) Hypoechoic solid 2 cm lesion, in the head of the pancreas, causing dilation of main pancreatic duct (5.6 mm). (b) EUS-FNA of the pancreatic lesion, using a 25 gauge needle.

sive, well-tolerated, operator-dependent procedure. The addition of fine-needle techniques provides samples for cytopathological analysis, which represents a major advantage over other imaging techniques (Fig. 1a and b).

Hewitt et al. recently published a meta-analysis assessing the diagnostic capability of EUS-guided tissue acquisition in PC patients [57]. Thirty-three studies published between 1997 and 2009 were included, with a total of 4984 patients, using a reference standard of definitive surgical histology or clinical follow-up of at least 6 months. Cytology results were classified as inadequate, benign, atypical, suspicious or malignant. The pooled sensitivity for malignant cytology was 85% and pooled specificity was 98%. If atypical and suspicious cytology results were included to determine true neoplasms, the sensitivity increased to 91% and the specificity was reduced to 94%. The diagnostic accuracy of EUS-guided tissue acquisition was enhanced in prospective, multicenter studies. Taking these results into account, the authors concluded that EUS-guided tissue acquisition should be considered when algorithms for investigating solid pancreatic lesions are planned.

Diagnostic accuracy based solely in imaging studies remains unsatisfactory, with nearly 10% of resected specimens considered preoperatively as PC being other lesions in the pathological exam of the surgical specimens. These are the so-called PC “mimickers” and include benign lesions, such as focal chronic pancreatitis, autoimmune pancreatitis, pancreatic tuberculosis and malignant ones, like

pancreatic lymphoma and metastasis from other organs’ neoplasia [68–71]. This becomes especially important as overall mortality after pancreatic surgery ranges from 0 to 10% and morbidity from 10 to 60% [72,73]. Therefore, an EUS-guided tissue acquisition with a diagnosis of a PC “mimicker” can spare patients from unnecessary surgery.

EUS-guided tissue acquisition is considered a safe procedure. The most common complication is mild-to-moderate pancreatitis which occurs in 0–3.4% of cases [45,51,74,75]. Rare but serious complications have been reported such as rupture of pseudo-aneurysm, severe bleeding and perforation, formation of pancreatic pseudocyst and abscess [51,76–78]. Bacteremia was an issue of concern due to the disruption of mucosal integrity occurs. Nonetheless, some studies showed that bacteremia rates in patients undergoing EUS-guided tissue acquisition were comparable to that of diagnostic endoscopies [79–81]. Taking into account that the frequency and severity of complications may vary from center to center and may be related to operator experience, Wang et al. conducted a systematic review to assess the morbidity and mortality associated with EUS-guided tissue acquisition [75]. Fifty-one articles with a total of 10,941 patients were included. The overall rate of EUS-guided tissue acquisition specific morbidity and mortality were 0.98% and 0.02%, respectively. In the small group of patients with complications of any kind, mild-to-moderate pancreatitis and post procedure pain were the more frequent events.

Another important advantage of a preoperative definitive diagnosis of malignancy is its role in the patient-doctor relationship. In fact, and even considering the nowadays “acceptable” rates of surgical morbidity and mortality, in high volume centers, from the patient’s and surgeon’s point of view, a biopsy proof of malignancy increases surgeon’s confidence and patients’ conviction of the urgent need of a demanding and often complex surgery.

Finally, the disseminated use of EUS-guided tissue acquisition in suspected pancreatic tumors can contribute for upcoming research, allowing the creation of a biobank. In this way, for example, in the near future, novel biomarkers could be discovered with the ability to detect PC in early phases or be used in testing new therapeutic agents [27,82].

5. Summary

EUS is undoubtedly an important tool to diagnose and stage PC and is often used complementary to MDCT. EUS-guided tissue acquisition is a safe procedure and the method of choice to sample pancreatic lesions with high diagnostic accuracy. The fear of tumor spread has limited its use in the past, but recent studies have demonstrated that EUS-guided tissue acquisition is a safe procedure, with rare and mild complications, and have contributed to the spreading of this technique in PC management algorithm [4,55,67]. EUS-guided tissue acquisition is especially useful in atypical and doubtful situations and in patients with high surgical risk, as it can avoid surgery and its associated morbimortality in lesions that can mimic PC, such as autoimmune pancreatitis or lymphoma. A biopsy proving malignancy increases the certainty of the indication of a surgical treatment, facilitating the surgeon-patient dialogue. Having EUS-guided tissue acquisition made its proof of concept when performed preoperatively to determine the definitive diagnosis, improving staging and supporting the correct indication for surgical resection of a pancreatic tumor, why should it not be considered in suspected early PC?

Conflicts of interest

None declared.

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3.3.3.

PAPER 3

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MINI REVIEW

Endoscopic ultrasound in pancreatic cancer treatment: Facts and hopes



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Summary Pancreatic ductal adenocarcinoma is one of the most common causes of cancer-related deaths. Since most patients present with advanced disease, its prognosis is dismal. New and more effective therapeutic strategies are needed. Endoscopic ultrasound is currently an indispensable tool for the diagnosis and staging of pancreatic ductal adenocarcinoma. In recent years, endoscopic ultrasound has evolved to become also a therapeutic procedure. On one hand, the role of endoscopic ultrasound in the management of pancreatic cancer-related symptoms (pain, obstructive jaundice, and gastric outlet obstruction) is now well established. On the other hand, its use as a mean to the delivery of anti-tumor therapies (injecting anti-tumor agents, assisting in radiotherapy, and guiding ablative therapies) is still mostly experimental, despite growing evidence supporting its feasibility, safety and efficacy.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth cause of cancer-related deaths in the United States (US) [1]. Its incidence is on the rise, while mortality rates have remained relatively unchanged [2]. It is forecasted that it will become the second leading cause of cancer-related death within a couple of decades [3]. Overall 5-year sur-

vival rate is 6% in the US [4]. The low survival rate stems mostly from the late stage at which most patients are diagnosed [5]. Only 15–20% of patients are amenable for initial surgical resection [4], with the majority of patients receiving either palliative chemotherapy or best supportive care [6]. Thus, new and more effective therapeutic strategies for patients with PDAC are urgently needed.

Since the introduction of endoscopic ultrasound (EUS)-guided fine needle aspiration (FNA) in the 1990s, it has become a mainstay in assisting in the diagnosis and staging of PDAC. Over the last few decades, however, EUS has evolved from a diagnostic tool to a minimally invasive therapeutic alternative to radiologic and surgical procedures for pancreatic diseases, including PDAC. Current evidence supports its use in the management of symptoms related

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to PDAC. In addition, growing data suggests its efficacy for the administration of anti-tumor therapies. In this comprehensive review we sought to evaluate the current and emerging roles of therapeutic EUS in management of patients with PDAC. The English literature was searched up to December 2018 and was conducted using PubMed, Scopus, Medline, ISI Web of knowledge and Google using the terms "endoscopic ultrasound", "pancreatic adenocarcinoma", "pain", "obstructive jaundice", "gastric outlet obstruction" and "tumour targeted therapies". Recursive searches, cross-referencing, and hand-searches of article reference lists were performed.

Treatment of cancer-related symptoms

Pain

Pain is an important symptom in PDAC, with some 75% of patients experiencing significant pain in advanced stages of the disease, with a dramatic impact in their quality of life [7]. Management of pain is a key integral part of the comprehensive therapy strategy in the patients with PDAC. Celiac plexus neurolysis (CPN) and celiac plexus block (CPB) are considered first line adjuvant therapies for the management of pain in pancreatic disorders [6,8].

CPN denotes permanent chemical ablation of celiac plexus (CP) through the injection of alcohol- or phenol-based solution into the CP, whereas CPB refers to the disruption of afferent pain signals transmission from the pancreas typically involving the injection of the CP with a local anesthetic mixed with a corticosteroid [9]. CP interventions are performed via a gastric approach using EUS-guidance and have high success rates and relatively low complication rates. EUS-guided approach is preferred over percutaneous or surgical approaches since it is associated with fewer side effects [9].

Currently, there are three modalities of CP ablation interventions guided by EUS in the setting of PDAC: CPN, celiac ganglia neurolysis (CGN), and broad plexus neurolysis (BPN) [10]. In addition, implantation of iodine-125 seeds into the celiac ganglia guided by EUS has been shown to be feasible, safe and efficacious in an animal model [11].

In 1996, Wiersema [12] reported for the first time the safety and efficacy of EUS-guided CPN in patients with pain due to intra-abdominal malignancies. Later, Gunaratnam et al reported a series of 58 patients with advanced PDAC submitted to EUS-guided CPN; pain scores improved significantly two-weeks after the procedures [13]. A retrospective study including 47 patients reported that EUS-CPN was successful in 68% of cases [14]. More recently, Wyse et al reported the results of a randomized, double-blinded, controlled trial, in which 96 patients with recently diagnosed inoperable PDAC were assigned to early EUS-CPN or conventional pain management. Pain relief was greater at 1- and significantly greater at 3-months in the EUS-CPN group compared to the conventional pain management group [15]. Although opioid consumption was similar in both groups at 1-month, it tended to be lower at 3 months in the CPN group compared to the conventional management group [15]. The above studies show the efficacy of EUS-CPN in obtaining temporary pain relief, however, long lasting analgesia

following CPN is not always achieved. Refractory pain has been reported in over 50% of patients [16]. One study comprising 24 PDAC patients evaluated the success rate with repeated CPN. The study showed that repeated CPN did not provide the same degree of pain relief as the initial procedure [17].

Due to the limitations of the long-term efficacy of CPN, there has been an interest in the diffuse injection directly into the celiac ganglia. Rate of ganglia detection varies between 70 to 80% depending on the instrument and the operator [18]. An initial retrospective study reported a 94% efficacy at 2- and 4-weeks for EUS-CGN in 17 patients with unresectable PDAC [19]. Another study compared the efficacy of EUS-CGN ($n=40$) versus EUS-CPN ($n=24$) in the setting of PDAC [20]. Pain relief was higher in the EUS-CGN group (65%) compared to the EUS-CPN-group (25%) [20]. More recently, a randomized study confirmed the superiority of EUS-CGN. This study, in which 34 patients were randomized to either EUS-CGN vs. EUS-CPN, showed that the former was associated with an efficacy that was significantly higher in the former than in the latter (73.5% vs. 43.5%, $P<0.05$) [21].

Lastly, a Japanese group recently reported the effectiveness of EUS-BPN that extends over the superior mesenteric artery (SMA) using a 25-gauge needle [22]. For that purpose, they have compared 34 patients submitted to EUS-BPN vs. 33 patients treated with EUS-CPN, and showed that EUS-BPN patients exhibited significantly greater reductions at days 7 and 30 visual analogue pain scale scores than EUS-CPN patients [22]. A recently published randomized double-blinded trial compared the efficacy of CGN versus that of CPN in patients with unresectable PDAC and abdominal pain [23]. Compared to CPN, CGN reduced median survival time without improving pain, quality-of-life, or adverse events [23].

In conclusion, EUS-guided celiac plexus intervention, in combination with conventional analgesia management, may be useful for pain management in patients of patients with PDAC. An earlier and more frequent use of such approach will definitely lead to a better quality-of-life of patients with PDAC experiencing pain. The more recently described approaches, through a broader blockage, may result in better response in selected cases; however, until further studies definitely prove their efficacy, they should be reserved for those cases in which conventional blockage fails.

Obstructive jaundice

Most patients with PDAC, particularly those arising from the head of the pancreas, present with bile duct obstruction. Endoscopic retrograde cholangiopacreatography (ERCP) is the preferred procedure for biliary drainage. However, ERCP failure occurs in 3–10 % of PDAC patients. In such cases, rescue options include percutaneous transhepatic drainage (PTBD), surgical interventions, and more recently EUS-guided biliary drainage (EUS-BD).

Following the first description the use of EUS-guided puncture of the bile duct for diagnostic purpose in 1996 [24], Giovannini et al reported a case of a patient with obstructive jaundice in whom a successful EUS-BD was performed after failed conventional ERCP [25]. Since then there are

over 1000 cases published in the literature, overall reporting technical and clinical success rates of 91% and 88% respectively [26].

Biliary access can be achieved via an intrahepatic or an extrahepatic route [26]. Technical and clinical success seems to be similar for both accesses, although the extrahepatic route seems to be safer [26]. After ductal access, biliary drainage can be achieved by three different approaches (Fig. 1):

- direct transluminal, either transgastrically (hepatico-gastrostomy) or transduodenally (choledochoduodenostomy), which involves dilation of the tract followed by stenting for transmural BD;
- rendezvous technique, in which intrahepatic or extrahepatic bile duct is punctured from the gastrointestinal tract, a guidewire is then advanced into the lumen of the duodenum via the papilla, allowing retrograde biliary intervention [26];
- antegrade, in which a stent is placed across the papilla, usually after passing a guide-wire via a transduodenal approach [27,28].

An initial prospective randomized study compared the success and efficacy of EUS-BD vs. PTBD [29]. Twenty-five patients were randomized (13 in the EUS-BD group and 12 in the PTBD group). All procedures were technically and clinically successful in both groups, and no differences were noted between the groups in terms of complication rates, cost or quality of life [29]. Conversely, two subsequent retrospective studies, comprising over 120 patients, suggested that EUS-BD is superior to PTBD in terms of safety, efficacy, and overall associated costs [30,31]. More recently, a multicentre, open-label, randomized trial compared EUS-BD vs PTBD in 66 patients with unresectable malignant distal biliary obstructions and failed primary ERCP [32]. Although technical, functional and clinical (quality of life) efficacy did not differ between the two groups, EUS-BD was associated with significantly fewer procedure-related adverse events, and significantly lower frequency of unscheduled re-interventions [32]. A retrospective expertise-based study from two centres reported that EUS-guided hepaticogastrostomy had similar success and adverse-event rates compared to PTBD, but associated with lower rates of re-intervention and length of hospitalization [33]. Lastly, the superiority of EUS-BD over PTBD has been recently confirmed in a meta-analysis comprising 9 studies with 483 patients [34]. Although there were no differences in terms of technical success between the 2 groups, EUS-BD was associated with better clinical success, fewer adverse events, and lower rate of re-intervention [34]. Recently, the efficacy of a fully-covered lumen-apposing metal stent (LAMS, Hot AXIOS; Boston Scientific, Massachusetts, United States) for EUS-guided choledochoduodenostomy in PDAC was shown in two case reports [35,36]. In one case, it was performed through an uncovered metal duodenal stent [36]. Although the results are promising, particularly in the latter setting, further studies are warranted.

In conclusion, in cases of ERCP failure, EUS-BD may be preferred over PTBD. However, it should be performed only by an experienced endoscopist with expertise in both

ERCP and EUS, and at a high-volume center where surgery and radiology can provide support in case of adverse events.

Gastric outlet obstruction

Gastric outlet obstruction is a common late manifestation of PDAC. It can cause significant morbidity including severe weight loss, malnutrition, dehydration, and electrolyte abnormalities [37]. In PDAC patients, gastric outlet obstruction usually develops due to progression of the primary tumor causing extrinsic compression of the duodenum, but it can also be caused by lymphadenopathy, carcinomatosis, or liver metastases [37].

The main goal of palliative therapy in this setting is to establish a way by which patients can maintain their oral food intake, and consequently improve their quality of life [38]. Management of gastric outlet obstruction can be performed endoscopically, with placement of a gastroduodenal stent [38]. When endoscopic gastroduodenal stent placement is unsuccessful, bypass surgery is an option [37]. However, in poor surgical candidates, an EUS-guided approach may be considered [9]. In this procedure, a gastro-jejunal fistula is created by obtaining an access to the jejunum via EUS-guided needle, followed by placement of a LAMS [39,40]. The use of a double-balloon enteric tube to distend the jejunum at the EUS-guided needle puncture has also been reported [41]. Another approach to create a gastro-jejunal anastomosis consists on the use of magnetic compression devices [9]. Following the early report in the animal setting [42], two human studies have been reported. In the first study, 15 patients with malignant obstruction were submitted to endoscopic gastroenteric anastomosis using magnets and a yoyo stent [43]. Success rate was nearly 90%, and no immediate major complications were observed [43]. Subsequently, a prospective multicenter cohort study investigated the safety and efficacy of endoscopic creation of a gastroenteric anastomosis formed by magnetic compression and self-expandable metal stent placement [44]. Briefly, under fluoroscopic guidance, a jejunal magnet (14-mm diameter) was mounted on a catheter and advanced to the horizontal part of the duodenum, while a gastric magnet (16-mm diameter) was inserted in the stomach attached to the endoscope until mating with the previously placed jejunal magnet occurred. After 8 to 10 days, a gastroscopy was performed to identify the gastroenteric fistula and to remove the magnets. Subsequently, a self-expanding metal stent with a was delivered through the gastroenteric fistula. Although the procedure was successful in 66.7% of patients, the study was terminated after inclusion of 18 patients due to a serious adverse event (stent perforation) leading to the death of the patient. In addition, stent migration occurred in three patients [44].

Although the endoscopic magnetic gastroenteric anastomosis is a promising concept, it cannot yet be recommended for routine clinical use. Further developments in technique and prospective studies are warranted before it becomes a feasible alternative to surgery in high risk surgical candidates.

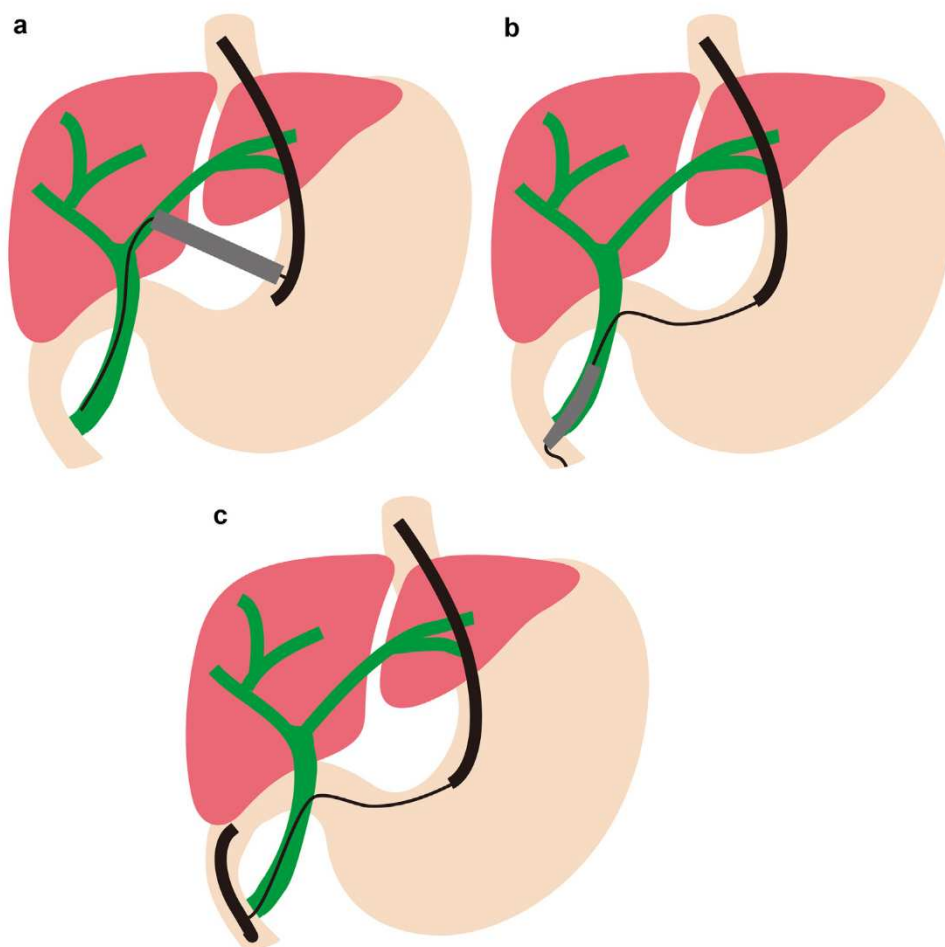


Figure 1 Different modalities of EUS-guided biliary drainage. When biliary drainage cannot be achieved by ERCP, an EUS-guided approach should be considered as a first line rescue option, given its advantages in relation to surgical derivation and percutaneous transhepatic procedures. EUS-guide biliary access can be obtained via an intrahepatic (through the stomach) or an extrahepatic (through the duodenum) route. After ductal access, biliary drainage can be achieved by three different approaches: a: direct trans-luminal, either transgastrically (hepatico-gastrostomy) or transduodenally (choledochoduodenostomy), which involves puncturing the biliary tree using a FNA needle, passing a guidewire, followed by dilation of the tract and stent insertion for a transmural drainage; b: antegrade, in which, after puncturing the common bile duct and successfully passing a guidewire to the duodenum, following track dilation, a stent is inserted inside the duct through the papilla; c: rendezvous technique, in which after intrahepatic or extrahepatic bile duct puncturing, a guidewire is advanced into the lumen of the duodenum through the papilla; subsequently the wire is captured and inserted retrogradely into the operating channel of a duodenoscope, allowing a retrograde biliary intervention (ERCP).

Tumor-targeted therapies

PDAC follows an aggressive clinical course, which is mirrored by complex underlying biologic features [45]. Hallmark pathogenic features of PDAC include oncogenic KRAS signaling, genomic instability, metabolic rewiring to support proliferation and survival, and a desmoplastic stroma with an immune-suppressive microenvironment [45]. The latter feature promotes tumor growth, is associated with the development of resistance to chemotherapy and radiotherapy, and constitutes a barrier to the delivery of therapeutic agents [46,47]. Since EUS offers real time dynamic images and a unique access to the pancreas, it has the hypothetical advantage of directly targeting the tumor through the desmoplastic stroma whilst minimizing systemic effects and complications [9]. This advantage has been recently

exploited as a mean to the delivery of anti-tumor agents, in assisting radiotherapy, and guiding ablative therapies (Table 1).

EUS-fine needle injection of anti-tumor agents

Progressive knowledge on the molecular pathogenesis of PDAC has directed increased attention toward studies on EUS-guided injection of chemical or biological agents for its treatment.

One of such examples is the injection guided by EUS of an allogeneic mixed lymphocyte culture (cytoimplant). Its administration induces potent cytokine secretion leading to the activation of the effector immune mechanisms, ultimately causing tumor regression [48]. A phase I trial

Table 1 Endoscopic ultrasound-guided tumor-targeted therapies for pancreatic adenocarcinoma.

Reference	Year	Therapy	Type of trial	Number of patients	Median survival (months)	Response
EUS-fine needle injection of anti-tumor agents						
Chang et al.	2000	Cytoimplant	I	8	13.2	3 partial response
Irisawa et al.	2007	Dendritic cells	I	7	10	1 complete response 3 partial response
Hirooka et al.	2009	Dendritic cells (+ gemcitabine)	I	5	16	1 partial response
Hecht et al.	2012	TNFERade	I/II	50	13.2	1 complete response 3 partial response
Herman et al.	2013	TNFERade	III	304	10	8 partial response
Hecht et al.	2003	ONYX-015	I/II	21	7.5	2 partial response
Hanna et al.	2012	BC-819	I/II	9	Not reported	3 partial response
EUS-assisted radiotherapy						
Sun et al.	2006	Brachytherapy	I	15	Not reported	4 partial response
Jin et al.	2008	Brachytherapy (+ chemoradiation)	I	22	Not reported	3 partial response
Saunders et al.	2010	SBRT	I	51	Not reported	Not reported
Park et al.	2010	SBRT	I	57	Not reported	Not reported
Khashab et al.	2017	SBRT	I	39	Not reported	Not reported
EUS-guided ablative techniques						
Song et al.	2016	RFA	I	6	Not reported	Not reported
Arcidiacono et al.	2012	RFA	I	22	6	6 partial response
Crino et al.	2018	RFA	I	8	Not reported	8 partial response

EUS: endoscopic ultrasound; SBRT: stereotactic body radiotherapy; RFA: radiofrequency ablation.

enrolled eight patients with advanced PDAC. Cytoimplant was obtained following the co-incubation of host and allogeneic healthy donor peripheral blood mononuclear cells, and then injected in escalating doses (3, 6, and 9 billion cells) into the pancreatic tumor by a single EUS-fine needle injection (EUS-FNI) [48]. No procedure-related complications were observed. The median survival was 13.2 months, with two partial and one minor responses [48]. Currently, there are no active clinical protocols evaluating cytoimplant for PDAC.

Dendritic cells (DCs) are the potent antigen-presenting cells, capable of priming naïve T cells and stimulating memory T cells to generate antigen-specific responses [49]. DCs have the capacity to activate T cells, interact with natural killer cells in tumor microenvironment, and directly kill tumor cells.

DC-based immunotherapy has been used in clinical trials for various cancers, including in PDAC. One pilot trial enrolled seven patients with advanced PDAC who had previously failed gemcitabine [50]. Patients received injection

of 10-billions of DC at two or three sites. No procedure-related complications were reported. Median survival was 10 months, with one complete and three partial responses [50]. In another pilot trial, five patients with advanced PDAC received a combination therapy with gemcitabine and OK432-pulsed DCs [51]. Median survival was 16 months, with two patients exhibiting sustained stable disease symptoms for over 6-months, with a further patient having a partial response [51].

Another immune strategy consists in the administration of tumor-necrosis factor (TNF)-erade (TNFERade™), a replication-deficient adenovirus carrying the human *TNF* gene. It is regulated by a radiation-inducible promoter [Egr-1] [52], which ensures maximal gene expression and subsequent TNF secretion to be constrained in space and time by radiation therapy, thus reducing the potential for systemic toxicity. In phase I/II trial, 50 patients with advanced PDAC were injected either percutaneously or under EUS-guidance with TNFERade™ along with systemic therapy with 5-fluorouracil and radiotherapy [53].

Median survival was 297 days; one and three showed complete and partial response respectively, while ten patients experienced stable disease symptoms. Interestingly, seven patients were eventually submitted to surgical resection, with six having clear margins, and three surviving for over two-years [53]. However, a later multicenter randomized phase III trial, which has included 304 patients with advanced PDAC, failed to show a survival benefit of combining TNFeradeTM with chemo and radiotherapy over chemoradiotherapy alone [54].

Another approach that is currently receiving attention in PDAC pertains to the use of oncolytic viruses (adenoviruses, herpesviruses, and reoviruses). Putative mechanisms of action include: viral replication cyclically within the cell causing its rupture, production of a cytotoxic protein, and/or inducing anti-tumor immunity [55]. One such example is ONYX-015, an engineered adenovirus lacking the *E1B* gene that preferentially replicates in malignant cells, leading to cell death [56]. Phase I/II trial included 21 patients with unresectable PDAC who received eight injections of ONYX-015 plus intravenous gemcitabine at the time of last injection [57]. Four serious adverse events were reported (sepsis in two, and duodenal perforation in another two). Median survival was 7.5 months, with two partial and two minor responses, and with further six patients showing stable disease symptoms [57]. Another example is the administration of HF10, a spontaneously mutated oncolytic virus derived from a herpes simplex virus-1, in combination with erlotinib and gemcitabine [58]. Phase I study showed this strategy was safe for treatment of locally advanced PDAC [58], suggesting that further large studies should explore the efficacy of combination therapy of HF10 with chemotherapy.

DNA plasmids have also been studied in the setting of PDAC, namely BC-819; it is DNA plasmid that targets the expression of diphtheria-toxin gene under the control of *H19* regulatory sequences. BC-819 has the potential to treat PDAC that overexpresses the *H19* gene [59]. EUS-guided or computed tomography (CT)-guided injection of BC-819 has been tested in a phase I/IIa trial, which included nine patients with advanced PDAC treated with concurrent chemoradiation [60]. No serious adverse events were reported. Three patients had partial response and two were successfully down staged and subsequently submitted to surgery [60].

Lastly, there is the possibility of delivering chemotherapy directly into the pancreas with EUS-guidance. In an animal model, paclitaxel bound to a thermosensitive gel carrier (OncoGel) was delivered to eight pigs. All animals tolerated well the procedure, and after two weeks high and sustained localized concentrations were detectable within 30 to 50 mm from the injection site [61].

Although EUS-fine needle injection of anti-tumor agents has not yet shown to significantly improve the survival in patients with PDAC, it seems that it can induce tumor cell death. Additional studies, however, are needed to further explore this therapeutic application in the future.

EUS-assisted radiotherapy

Radiotherapy is a therapeutic option for patients with locally advanced PDAC [6]. This group of patients is conventionally

offered fractional external beam radiation therapy combined with chemotherapy. Other therapeutic options are emerging such as brachytherapy and stereotactic body radiotherapy (SBRT) after fiducial placement [9].

Brachytherapy is a procedure that involves placing of a radioactive seed directly into the tumor. Iodine-125 is the most commonly used seed. EUS-guided brachytherapy was first reported in a pilot study in which 15 patients with locally advanced PDAC received brachytherapy without chemotherapy or external radiotherapy [62]. Local complications (pancreatitis and pseudocyst formation) occurred in three patients, while grade III hematologic toxicity occurred in other three patients. After a median follow-up time of 10.6 months, 27% and 20% of patients experienced partial and minimal respectively, whereas 33% of patients had stable disease. In addition, reduction in pain severity was documented in 30% of patients, though this effect was short-termed [62]. In another study, 22 patients received EUS-guided brachytherapy combined with gemcitabine plus 5-fluorouracil chemotherapy [63]. Although tumor growth was effectively controlled in 59.1% of patients and reduction in pain severity was noted in all patients, no significant survival benefit was observed [63].

SBRT allows a precise administration of radiotherapy, therefore minimising irradiation of adjacent normal tissue. Early studies have shown that SBRT is comparable with external beam therapy in terms of both local tumor control and overall survival [64–66]. Placement of fiducial markers prior to SBRT allows precise tumor targeting. Although traditionally placed by surgical or percutaneous approach, EUS seems ideal for placement of fiducial markers in PDAC. EUS-guided fiducial marker placement was first reported in 2006; in this study fiducial markers were successfully placed in six out of seven PDAC patients [67]. Later, two other studies, together including 106 patients, reported 88 and 90% successful rate, therefore confirming the feasibility and safety of EUS-guided fiducial marker placement [68,69]. More recently, Khashab et al. compared 2 types of commercially available fiducials (traditional vs. visicoil) [70]. Visibility score was significantly higher for traditional compared to visicoil fiducial, but no differences in terms of migration rates, technical success or complication rate were observed between the two [70].

EUS-guided ablative techniques

Radiofrequency ablation (RFA) is a well-established technique to ablate dysplastic and neoplastic tissue via local thermal coagulative necrosis [71]. It has been demonstrated to be feasible via open, laparoscopic or percutaneous approaches for locally advanced PDAC [72,73]. EUS-guided RFA offers the best combination of excellent visualization, real-time imaging guidance, precise localization, with minimal invasiveness. Previous studies have shown that EUS-RFA is feasible and safe for the porcine pancreas [74,75]. Recently, a small pilot study including six patients with unresectable PDAC showed that EUS-RFA was performed successfully in all six patients [76]. Side effects were reported in two patients who experienced mild abdominal pain [76]. One study was the feasibility and safety of the use of cryotherm probe, a flexible bipolar device that combines radiofrequency with cryogenic cooling. Amongst 22 patients

with unresectable stage III PDAC, the cryotherm probe was successfully performed in 16 (success rate 72.8%) [77]. No severe adverse events related to the procedure were observed. Median post-ablation survival was six months, and a reduction in tumor size was noted on follow-up CT in six out of sixteen patients [77]. A recent study reported the safety and efficacy EUS-RFA in 8 patients, showing that in all an ablated area inside the tumor was obtained. Although three patients reported minor post-procedural pain, no major adverse event was observed after a mean follow-up of 6 months [78].

Photodynamic therapy (PTD) is an approach where a needle placed in the target tissue is used to deliver a specific wavelength of light. Following activation by a photosensitizing agent, it produces localized tissue necrosis, through the generation of reactive oxygen species [79]. Although encouraging results of PDT have been reported in the setting of cholangiocarcinoma, its use in PDAC is still lagging behind. One study in an animal model showed that EUS-guided PDT is a safe and able to induce areas of focal tissue ablation within the pancreas [80]. More recently, a phase I/II study comprising fifteen patients with advanced stage PDAC demonstrated that PDT is a feasible and safe technique, allowing the induction of tumor necrosis [81].

Conclusion

EUS is an indispensable tool in the management of PDAC; it is useful not only for tissue diagnosis and staging, but also for therapeutic purposes. Some EUS-guided therapies, such as CP interventions for pain control and obstructive jaundice management after failed ERCP have become widely accepted, whereas other techniques, namely anti-tumor injection and ablative therapies, are still mostly experimental, and have yet to evolve before becoming routine practice. Data on the long-term efficacy of these new approaches, and large prospective randomized studies are needed to confirm its real clinical benefits. Given the lack of effective systemic treatments at present, continuous research in therapeutic EUS is warranted.

Disclosure of interest

The authors declare that they have no competing interest.

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Author's contributions

PMR — study concept and design, literature review, and draft of the manuscript; RL — literature review, and draft of the manuscript; GM — supervision.

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3.3.4.

PAPER 4

Moutinho-Ribeiro P, Macedo G, Melo SA.

**Pancreatic cancer diagnosis and management:
Has the time come to prick the bubble?**

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Pancreatic Cancer Diagnosis and Management: Has the Time Come to Prick the Bubble?

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Pancreatic cancer (PC) is associated with poor prognosis and very dismal survival rates. The most effective possibility of cure is tumor resection, which is only possible in about 15% of patients diagnosed at early stages of disease progression. Recent whole-genome sequencing studies pointed genetic alterations in 12 core signaling pathways in PC. These observations hint at the possibility that the initial mutation in PC might appear nearly 20 years before any symptoms occur, suggesting that a large window of opportunity may exist for early detection. Biomarkers with the potential to identify pre-neoplastic disease or very early stages of cancer are of great promise to improve patient survival. The concept of liquid biopsy refers to a minimally invasive sampling and analysis of liquid biomarkers that can be isolated from body fluids, primarily blood, urine and saliva. A myriad of circulating molecules may be useful as tumor markers, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTC), circulating tumor proteins, and extracellular vesicles, more specifically exosomes. In this review, we discuss with more detail the potential role of exosomes in several aspects related to PC, from initiation to tumor progression and its applicability in early detection and treatment. Exosomes are small circulating extracellular vesicles of 50–150 nm in diameter released from the plasma membrane by almost all cells and exhibit some advantages over other biomarkers. Exosomes are central players of intercellular communication and they have been implicated in a series of biological process, including tumorigenesis, migration and metastasis. Several exosomal microRNAs and proteins have been observed to distinguish PC from benign pancreatic diseases and healthy controls. Besides their possible role in diagnosis, understanding exosomes functions in cancer has clarified the importance of microenvironment in PC progression as well as its influence in proliferation, metastasis and resistance to chemotherapy. Increasing knowledge on cancer exosomes provides valuable insights on new therapeutic targets and can potentially open new strategies to treat this disease. Continuous research is needed to ascertain the reliability of using exosomes and their content as potential biomarkers, so that, hopefully, in the near future, they will provide the opportunity for early diagnosis, treatment intervention and increase survival of PC patients.

Keywords: pancreatic cancer, early diagnosis, liquid biopsy, biomarkers, exosomes

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), the most frequent type of pancreatic cancer (PC), is associated with poor prognosis, with 53,670 new cases and 43,090 estimated deaths in 2017 (1). It represents around 3% of the new cancer cases each year, but it is the fourth most common cause of cancer mortality (2). It is expected to become the second cause of death by cancer by 2020 in the USA (2). On the contrary to the death rates for many other cancer, such as lung, colorectal, breast, and prostate, the death rate for PDAC patients has increased (3) as well as its incidence, that has raised in about 30% (2).

Pancreatic cancer is typically asymptomatic in its first stages of development, and as a consequence of the late diagnosis, this disease presents a very low survival rate. Combining all stages, the overall 5-year survival rate is 5% (4). Tumor resection is the only possibility of cure, but recurrence often happens and, therefore, the 5-year survival rate in resected patients is only up to 25% (1). Its unique tumor biology contributes to early recurrence, metastasis, and a subpar response to conventional therapies (5).

Since lifetime risk of PC in general population is low (1.3%), a population-based screening is not recommended (6). Meanwhile, individuals with family history or genetic predisposition have been identified as risk groups. According to the International Cancer of the Pancreas Screening Consortium (CAPS) it has been proposed that these individuals at higher risk for PC should be considered for screening (6, 7). The referred Consortium defined eligible individuals to be those with more than 5% of lifetime risk or with a 5-fold increased relative risk for PC (6, 7). Nonetheless, this group of patients represents only 10% of the total spectrum of PC, the other 90% of cases being sporadic (8).

PANCREATIC CANCER PRECURSOR LESIONS: A WINDOW FOR OPPORTUNITY

Recent studies based on whole-genome sequencing indicated 12 important signaling pathways altered in PC. It was also suggested that the initial mutation occurred nearly 20 years before the first symptoms. This evidence has offered a time frame for pancreatic carcinogenesis, suggesting that a large window of opportunity may exist for early detection, which could improve the prognosis of this lethal disease (9, 10).

In recent years, a myriad of biomarkers have been investigated with specificity for PC detection (8). The ideal biomarker should be able to detect the disease in its early stages, when patients are still amenable for a curative treatment, or even in a more favorable scenario, its premalignant precursor lesions. Pancreatic cancer precursor lesions are intraductal papillary mucinous neoplasms (IPMNs), the mucinous cystic neoplasms (MCNs), and the pancreatic intraepithelial neoplasias (PanINs), the first two being macroscopic cystic alterations and the last one representing microscopic non-invasive epithelial proliferations within the pancreatic ducts (5). Intraductal papillary mucinous neoplasms (IPMNs) are papillary proliferations inside the pancreatic ducts that usually secrete thick mucus, leading to its focal dilation. They represent up to 10% of the neoplasms

of the pancreas, and the ones harboring high-grade dysplasia carry an important risk of malignant transformation (11, 12). Depending on the extent of ductal involvement, three subtypes can be recognized: the main-duct intraductal papillary mucinous neoplasm (MD-IPMN), the branch duct intraductal papillary mucinous neoplasm (BD-IPMN), and the mixed type. Malignant transformation is more frequent in main duct and mixed types IPMNs, while BD-IPMNs are considered indolent lesions (11, 12).

In relation to cytoarchitectural and immunophenotypic features, four histological subtypes of IPMNs are considered: the gastric type (49–63%), the intestinal type (18–36%), the pancreaticobiliary type (7–18%), and the oncocytic (1–8%) type (11, 13). Main-duct intraductal papillary mucinous neoplasm (MD-IPMNs) are more frequently of the intestinal type, a combination that carries the highest risk of invasive transformation, usually giving rise to a colloid type carcinoma. On the other hand, the branch duct intraductal papillary mucinous neoplasm (BD-IPMN) are predominantly of the gastric type, which are characterized by an insignificant risk of malignant transformation. The pancreaticobiliary type usually harbors high-grade dysplasia and is considered the aggressive evolution of the gastric type. Finally, the rarer oncocytic type presents with cytological atypia, frequently with high-grade dysplasia (14).

MCNs, unlike IPMNs, are neoplasms that develop in the pancreatic parenchyma, without involvement of the ductal system. Characteristically they are seen in women, rarely occurring in men, with a preferential location in the body and tail of the gland (15).

An ovarian-type stroma is an essential feature for the diagnosis of MCNs that clearly separates them from the much more frequent IPMNs. Depending on the grade of cytologic atypia in epithelial lining (low, intermediate or high-grade dysplasia), which has been associated with point mutations in KRAS and p53 genes, mucinous cystic neoplasms (MCNs) may exhibit different risks of malignant potential (15–17). Interestingly, the observation that in invasive MCNs, the inactivation of SMAD4/DPC4 suppressor gene complex occurs only in the epithelial lining but not at the stroma level, suggests that the typical ovarian-type stroma of these lesions is not involved in the process of malignant transformation (18). The reported incidence of invasive carcinoma among MCNs is variable according to different series, ranging from 6 to 36% (15, 19, 20).

With the development and widespread of imaging modalities, the diagnosis of both of these macroscopic cystic PC precursor lesions is increasing. As the majority of them are asymptomatic and discovered incidentally, its proper management is not consensual and is a matter of debate by many International Societies worldwide. In fact, despite the availability of a significant number of guidelines and recommendations (21), there is still a lack of consensus in the decision of which one(s) to follow.

The vast majority of carcinomas originate from microscopic non-invasive epithelial proliferations within the pancreatic ducts, described as PanIN (22). These lesions are considered precursors

in the stepwise progression from intraepithelial to invasive neoplasia. This morphological progression is accompanied by accumulation of genetic changes, in which activating KRAS mutations are thought to be the driving force (8, 12). PanIN lesions are characteristically asymptomatic and are composed of columnar to cuboidal cells with varying amounts of mucin and different degrees of cytological and architectural atypia (23). These lesions are classified into 3 grades: PanIN-1A (flat) and PanIN-1B (papillary) are low-grade lesions with minimal cytological and architectural atypia, while high-grade PanINs (PanIN-3), also described as “carcinoma *in situ*,” are characterized by severe cytological and architectural atypia (23). In cadavers of patients over the age of 80 the prevalence of PanIN lesions is about 55%. Also, these lesions are very frequent in patients with concomitant PC and in familial PC kindreds. The major problem concerning these very frequent lesions is their identification and the evaluation of its malignant potential. Currently, there is no imaging technique capable of an accurate diagnosis of a PanIN lesion and many promising biomarkers are being investigated for this purpose.

CONVENTIONAL DIAGNOSTIC TOOLS ARE INSUFFICIENT FOR EARLY DETECTION

Early diagnosis of PC is very challenging with the currently available methods (**Figure 1**) (24). Unlike colonoscopies for colorectal cancer and serum prostate-specific antigen (PSA) levels for prostate cancer, there is currently no standardized PC screening strategy, even for high-risk populations. Pancreatic cancer diagnosis and staging depends, substantially, on imaging modalities including ultrasonography (US) and endoscopic ultrasonography (EUS), multidetector computed tomography (MDCT), magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP), endoscopic retrograde cholangiopancreatography (ERCP), and positron emission tomography (PET) (25–30).

Nowadays, multidetector computed tomography (MDCT) is the imaging technique of choice for pancreatic diseases, especially in the setting of solid tumors, where it has high accuracy to detect and to stage pancreatic malignancies (31). However, its sensitivity may be suboptimal as it misses some target lesions in the PC screening context. Even when considering thin-section, triple-phase helical CT, the sensitivity to detect lesions smaller than 2 cm is only up to 80% (32). Moreover, ionizing radiation exposition is also an important drawback, precluding CT to be an ideal screening and/or surveillance imaging technique.

In high-risk individuals, magnetic resonance imaging (MRI) can be used as a non-invasive screening imaging test considering the possibility to scan the entire abdomen and pelvis, avoiding radiation exposure. Considering the MRCP, this technique offers, in a non-invasive way (in contrast to ERCP), the capacity to characterize the ductal anatomy of the pancreas and diagnose small cystic lesions such as IPMNs. Preliminary data from CAPS3 study, that included high-risk patients submitted to surgical resection, suggests that MRI/MRCP may be superior to CT particularly for detection of IPMNs (71 vs. 14%, $p < 0.001$) (33).

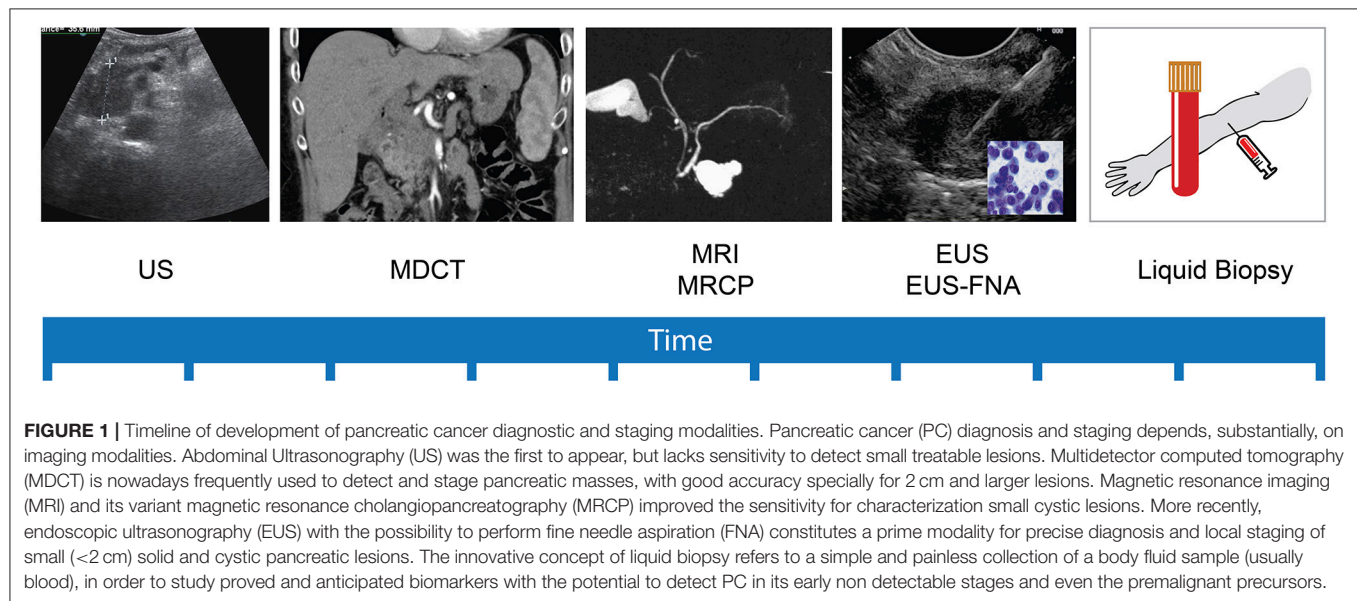
Some MRI features have been recently added to this technique improving its diagnostic capacity. In fact, diffusion-weighted imaging (DWI), a technique based on the Brownian motion of water molecules in tissue (34), has brought functional aspects into conventional anatomic evaluation, allowing higher contrast resolution and the identification of very small PC lesions.

In diagnosing PC, the advantage of EUS over MDCT has been reported for more than 10 years (35, 36). Despite being an imaging technique operator-dependent, with inherent risks of invasiveness and sedation, EUS has progressively being considered as the most accurate tool to investigate pancreatic diseases. It is also a non-radiation technique that can offer high-resolution images and can accurately characterize solid and cystic lesions. Moreover, it can evaluate cystic wall features associated with increased risk of malignancy, namely mural nodules, and other focal thickenings. The data analysis from a screening program involving high-risk individuals that were submitted to surgical resection confirmed the superiority of EUS, as it was able to detect almost twice as many neoplastic lesions comparing to MRI/MRCP or CT (33). Also, past studies (37, 38) that used EUS as a screening tool showed its accuracy in detecting asymptomatic precancerous branch duct IPMNs, large PanINs, incidental pancreatic endocrine tumors and ductal adenocarcinomas. The chance to add some recent techniques such as EUS-guided elastography and contrast-enhanced imaging has expanded new and promising fields of investigation (37–40).

Considering EUS-guided fine-needle aspiration (EUS-FNA), when performed in suspected lesions, it shows diagnostic accuracy for malignancy of more than 85–90%. Apart from its role in the study of solid lesions, the investigation of pancreatic cystic lesions by EUS-FNA can also be very useful, allowing a cytological diagnosis of IPMN and MCN in up to 70% of the cases (41). “Cell-block preparation” and “core tissue sampling” are two developments of this technique (42), which might be useful not only in providing more material for histological evaluation, but also for recently developed ancillary diagnostic techniques, namely: microRNA profiling, KRAS mutation detection and chemo sensitivity testing (43–45).

Lately, confocal laser endomicroscopy has surged as a technological improvement to EUS with a particular interest in cystic lesions characterization. In this technique, a dedicated miniprobe is introduced through a 19-gauge needle previously inserted into the cystic lesion, allowing a real time direct visualization of the epithelial lining at a microscopic level, permitting the identification of suspicious architectural changes (46).

Nowadays, no biomarker exists with adequate sensitivity and specificity for routine clinical diagnosis or screening of PC (8). Carbohydrate antigen (CA) 19-9 and carcinoembryonic antigen (CEA) are the most commonly used blood-based tumor biomarkers in clinical practice. The only biomarker approved by the US FDA for monitoring the progression and therapeutic response of PC is CA 19-9, which is also the only one recommended by the NCCN guidelines for clinical management of PC patients (47). Nevertheless, it is not specific and can be found in normal pancreatic and biliary duct cells, as well as in gastric, endometrial, colonic, and salivary epithelia. In



fact, depending on the series, its specificity ranges from 30 to 100% (48–50), so the extremely high rate of false positive results prohibits its routine use for diagnosis. Moreover, CA 19-9 sensitivity is also imperfect, ranging from 41 to 86% (48–50). Additionally, up to 15% of general population do not express Lewis antigen and, consequently, CA 19-9 levels cannot be measured (50–53). Another clinically relevant issue is the fact that only 65% of patients with resectable tumors have increased levels of CA 19-9. In relation to its ability to differentiate PC from chronic pancreatitis, this biomarker is also inadequate, as up to 40% of patients harboring this last condition exhibit CA 19-9 levels above the normal range (52, 54). Considering all these pitfalls, serum CA 19-9 is used primarily as a prognostic tool in monitoring patients for recurrence or managing those with late stage disease (52, 55).

Taking the aforementioned aspects in account, it is understandable that most patients present with advanced disease, with only up to 25% having resectable tumors (56–58). We should also have in mind that even if these 25% of patients are identified and properly treated under the current standards of care, their survival, at the best, is up to 24 months (4). So, the focus should be, on one side, in aiming to detect the “real” curable lesions, that is the premalignant and the very small malignant ones, and, on the other side, the development of better therapeutic options. For the early detection scenario, the conventional diagnostic tools are far from being competent. In fact, available non-biopsy tests (serum CA19-9) and imaging techniques lack the sensitivity and specificity necessary to detect tumors smaller than 1 cm in the context of a 1.5% lifetime risk disease (59–65).

In consequence, it is crucial to develop new and improved strategies which can overtake all the obstacles described and diagnose primary tumors that can be resectable at very early stage. The development of markers with high sensitivities and specificities for PC and for its precursor conditions should be a

priority issue, with extreme importance mainly in the context of high-risk individuals (66).

LIQUID BIOPSY, A HORIZON FOR EARLY DETECTION AND SURVIVAL IMPROVEMENT

As mentioned before, given the typical late stage of disease at the time of presentation, when treatment is disappointing, PC remains one of the most dismal diseases worldwide, with incidence nearly parallel to mortality. There has been much effort invested in identifying accurate tumor markers, ideally present in the timeframe between cancer onset and invasion, to allow diagnosing PC in early curable stages, to ultimately improve patient's survival (67, 68). In this setting, the ideal biomarker should be easily detected with satisfactory sensitivity and specificity and should distinguish PC from other benign pancreatic lesions. In the context of early detection, the identification of preneoplastic conditions, such as PanINs, IPMNs and MCNs, is of great importance (69).

The concept of liquid biopsy refers to the analysis of biomarkers present in a sample of a body fluid collected through a simple and painless, minimally invasive technique. The body fluids mostly used for biomarker isolation are essentially blood, urine and saliva (70–72). A myriad of circulating molecules may be used as tumor markers, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTC), circulating tumor proteins, and extracellular vesicles, more specifically exosomes (66, 73). Blood is easily accessible and relatively stable, making serum an ideal specimen to explore potential biomarkers. However, biomarkers secreted into serum are extremely diluted and probably obscured by other more-abundant serum proteins (74). Technological advances in the last decade have provided more opportunities to discover circulating biomarkers based

on “omics” analyses, including methods focused on proteins, nucleic acids, CTCs, and exosomes. Numerous proteins of low abundance can be analyzed by mass spectrometry-based approaches and proteomic technologies (24).

In recent years, based on the expression of transcriptional profiles and structural variations, different molecular subtypes of PC have been described through genomic analyses (75–78). The early detection of mutant genes that identify PC and its subtypes is essential for an effective strategy for the management of the disease.

In PDAC, there are four major driver genes (one oncogene and three tumor suppressor genes) implicated in tumorigenesis (5). KRAS is the most frequently altered oncogene that encodes a GTPase which mediates downstream signaling from growth factor receptors; somatic mutations, clustered in specific hotspots (most in codon 12), are identified in more than 90% of PC (79). CDKN2A, by turn, is the most frequently altered tumor suppressor gene, with loss of function in more than 90% of tumors; it encodes an important cell-cycle regulator (79). TP53 is another tumor suppressor gene, with essential role in cellular response to stress, also exhibits frequent somatic mutations (79). Lastly, SMAD4, a tumor suppressor gene mediating downstream signaling of the transforming growth factor β (TGF β) receptor is inactivated in about half of the PC cases (79).

Since Mandel and Metais, in 1948, (80) first described circulating free DNA in body fluids, an exponential interest in non-invasive technology for disease monitoring has been the focus of research in many centers worldwide.

Recently, due to the possibility to pair genomic tests with tests on CTCs, circulating tumor nucleic acids (ctNAs) and tumor-derived exosomes, liquid biopsies have gained increased value for clinical application (71, 81, 82).

During PC initiation and progression, many different genetic modifications take place, including genetic diversification, amplifications and homozygous deletions, an increase in duplicate chromosomal number, recapitulation of clonal expansion, clonal selection, driver mutations and losses of heterozygosity (10, 83–90).

Next-generation sequencing techniques provide deeper insight into somatic mutations and epigenetics analysis of the genome and broaden the characterization of circulating tumor DNA (ctDNA) and (cfRNA). With the development of cell tracking techniques and flow cytometry, it is now possible to capture and analyze CTCs and exosomes (24).

In this manner, the capabilities of liquid biopsy are enormous, allowing the characterization of tumor biomarkers in the same way tissue biopsy does, favoring improvement of the knowledge of tumor heterogeneity and, most importantly, contributing to early detection, monitoring of disease progression and response to treatment (**Figure 2**) (71, 82, 91–96).

EXOSOMES: THE BUBBLES OF THE FUTURE?

Exosomes are small cup-shaped extracellular vesicles (50–150 nm in size) released from the plasma membrane by almost all cells,

including cancer cells (**Figure 1**) (24). They play an important role in intercellular communication, tumorigenesis and cancer metastasis (97–100). Structurally, they are enveloped by a lipid bilayer membrane with tissue-specific content instead of cellular organelles, such as pathogenic mRNA, microRNA, DNA fragments, and proteins (101, 102). After release, exosomes are stable in the extracellular environment or enter the circulation and can be taken up by neighbor or distant cells (103, 104). Exosomes allow the exchange of material and information between cells, altering gene expression or mediating RNA silencing (105).

Considering their extensive distribution and functions, exosomes are ideal candidates to find circulating biomarkers for PC detection and management (67, 104, 106). Moreover, exosomes have some advantages over other biomarkers (106, 107). First, they are widely distributed in nearly all body fluids, including serum, and are relatively stable when stored for long term at -80°C . Second, cancer cells secrete more exosomes than normal healthy cells. One of the reasons for this can be the acidic conditions of the tumor microenvironment that enhances the release of exosomes (108–110). Third, exosomes contain a combination of proteins, DNA, coding and non-coding RNAs, and lipids that can be used as a natural panel of biomarkers for simultaneous evaluation. Fourth, PC-derived exosomes enter the circulation at an early stage of cancer development and are related to metastasis. In fact, on the contrary of some other cancer markers that are released into the blood only after necrosis-related cell death occurs, a phenomenon usually associated with advanced stage disease with large volume tumor mass, cell-secreted exosomal nucleic acids (DNA fragments, mRNA, miRNAs, and others) are released in circulation during the initial phases of the tumorigenesis process (66, 91, 107). This aspect is critical for the early detection of PC because PC cells are able to metastasize at an early stage, with great impact on prognosis.

Recent studies have implicated PC-derived exosomes in the early development of PC (103) and showed that they contribute for establishing a premetastatic niche in the liver (98, 111) and that they can promote tumor formation and proliferation (112). In relation to exosomes' content, a main focus of research has been on RNA and microRNA profiling, in part due to the established utility of a variety of some of these molecules in cancer screening and also due to the relative ease feasibility of its amplification (99, 113–118). By turn, in order to determine their cellular origin, exosomal proteomic profiling is being an important focus of research in recent years (114).

Besides their possible role in diagnosis, the study of exosomal function has contributed to the improvement of the comprehension of the microenvironment related to PC and progression of the disease. The ultimate aim of understanding the way exosomes can influence tumor initiation, proliferation and metastasis is improving the knowledge on PC pathophysiology and patient prognosis. Moreover, its role in the development of some paraneoplastic conditions, such as diabetes *mellitus* and cachexia, and in the resistance to chemotherapy, can provide insights for future therapeutic targets (119–125).

The extraction of exosomes from body fluids has been described using various methods and technologies. Commonly

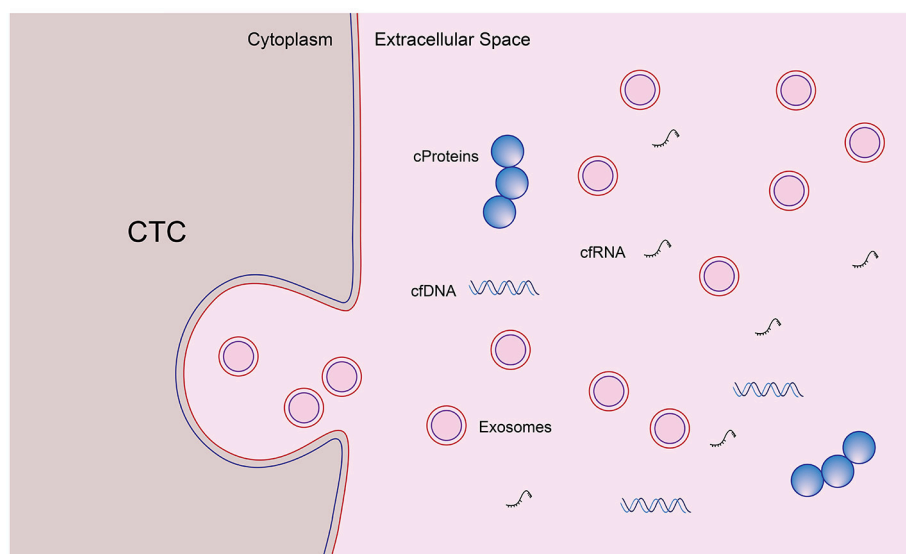


FIGURE 2 | Current understanding of molecular biomarkers for pancreatic cancer. A myriad of circulating molecules may be used as tumor markers, including cell-free DNA (cfDNA), cell-free RNA (cfRNA), circulating tumor cells (CTC), circulating tumor proteins, and extracellular vesicles, more specifically exosomes. The characterization of these tumor biomarkers favors improvement of the knowledge of tumor heterogeneity and, most importantly, contributes to early detection, monitoring of disease progression and response to treatment. Moreover, some of these molecules, in the near future, can play a role as therapeutic targets, hopefully allowing the control of the disease during its early curable stages.

used methods for isolation are ultracentrifugation, precipitation, size, immunoaffinity, and microfluidics (126).

Strong efforts have focused on developing sensitive diagnostics tools improving early detection of PC via identifying pancreatic cancer-associated exosomal markers (67, 104, 127). The first case-control study on PC exosomes was conducted by Que et al. (128), where four exosomal microRNAs were evaluated as candidates. In this study, a moderate discrimination of cases from controls was seen with miR-21 and miR-17-5p. In 2015, in a study involving patients with PC, chronic pancreatitis and controls, a higher expression of exosomal miR-10b was shown only in cancer patients (129). Although this cohort was a small one, the strength of this study was the use of new technology of label-free nanoplasmonic-based short non-coding RNA sensing. In the same year, a more extended cohort including PC patients, patients with chronic pancreatitis, individuals with benign pancreatic neoplasms and controls was evaluated by Madhavan et al. (130). In this well conducted study, a combination of four miRNAs (miR-1246, miR-4644, miR-3976, and miR-4306) and five proteins (CD44v6, Tspan8, EpCAM, MET and CD104) in circulating exosomes was able to distinguish PC patients from non-cases ones, with a sensitivity of 1.0 (CI: 0.95-1) and a specificity of 0.80 (CI: 0.67-0.90).

In the past years, the number of exosomal miRNAs studied in this context was considerable. Examples of these molecules are miR-21, miR-17-5p, miR-155, miR-34, miR-196a, miR-181a, miR-181b, miR-138-5p, miR-494, miR-542-3p, miR-31, and miR-205, that have been implicated for several studies to have the capacity, when upregulated, to promote cellular proliferation and angiogenesis, metastasis and disease progression and even chemo-resistance in PC patients (113, 128, 131-138).

These studies emphasize the importance of exosomal miRNAs not only as diagnostic and prognostic biomarkers, but also open a field of investigation of its use as potential targets for treatment PC.

Recently, Melo et al. showed that glypican-1 (GPC1), a membrane anchored protein, in circulating exosomes may distinguish with 100% specificity patients with PC or precancerous pancreatic lesions from patients with benign pancreatic diseases (139). Melo et al. reported that GPC1 expression patterns in exosomes secreted by PC could be utilized to identify subjects with PC early and offer considerable insights into the disease progress and tumor load. A comparison of exosomes from PC and control cell lines indicated that the exosomes from cancer exhibited enhanced levels of GPC1. In serum specimens from subjects with PC ($n = 190$), a significantly larger amount of GPC1+ circulating exosomes was present compared with normal controls ($n = 100$). Interestingly, direct analysis of GPC1 in serum itself revealed lower sensitivity and specificity than measurement of GPC1 in purified serum exosomes. Furthermore, GPC1+ exosomes were also confirmed to contain identical KRAS mutations, which frequently are present in PC and precancerous lesions and have been considered a fundamental mutation (139). Moreover, higher levels of GPC1 positive circulating exosomes were seen in both PC and PC precursor lesions, such as IPMNs, when compared to other benign diseases of the pancreas and healthy controls, with a perfect area under the receiver-operating characteristic curve (AUC) of 1.0. The authors concluded that given the high sensitivity and specificity of exosomal GPC1 in differentiating PC, independently of its stage, from non-PC controls, this biomarker could have a promising role

in PC early detection. Not surprisingly, when compared to the tumor marker CA 19-9 (AUC: ranging between 0.69 and 0.79), exosomal GPC1 was found to be significantly superior ($p < 0.001$) (139). Additionally, using a genetically engineered mouse that progressively developed into PC, the identification of GPC1+ exosomes exhibited positive results prior to pancreatic lesions being detectable by MRI. Tumor burden was associated positively with levels of GPC1+ circulating exosomes. In most subjects, the exosome levels reduced following the removal of the solid tumor. In this manner, circulating exosomal GPC1 may be seen as a prognostic indicator too, reflecting tumor load and monitoring disease progression and patients' survival. Moreover, evolving research will address if this biomarker can play a role in the treatment of PC as a potential pharmacological target (139). Many others since then have reported findings related to GPC1 detection in exosomes, not only in PC but also breast and colorectal cancer cases (140–148). In any event, in the future, further independent confirmation of exosomal GPC1 performance is needed, assessing its role as a more reliable marker in predicting diagnosis and prognosis of PC when compared to CA19-9, but also, and as it seems to be overexpressed in some precursor lesions such as IPMNs, its potential role in individualizing the management of such conditions (149).

Also interestingly, a paper from Hoshino et al. (98) has described that integrin $\alpha v \beta 5$ -expressing PC exosomes can determine liver metastasis and that targeting that molecule reduced exosome absorption by resident cells and inhibited liver involvement. The levels of integrin $\alpha v \beta 5$ were noticeably enhanced in exosomes isolated from PC individuals with liver metastasis compared with those with no distant metastasis. Together, these findings have raised the possibility that GPC1+ and integrin $\alpha v \beta 5$ -expressing circulating exosomes may be used as indicators of PC progression and liver metastatization.

Taking in account the excellent biodistribution and biocompatibility of exosomes, the idea of its utilization as vehicles for drug, genes or nucleic acids delivery has gained increased acceptance for continuous research in the field of PC treatment (124, 150, 151), especially when considering the fact that they can be particularly targeted to specific cell types by engineered exosomes-producer cells (124, 150, 152). This is important especially when dealing with a tumor with such difficulties in therapy delivery due to the intrinsic resistance of its microenvironment and its dense stroma (153).

CONCLUSION AND NEW PATHWAYS OF INVESTIGATION

Given the timeframe from pancreatic tumor initiation to invasion and metastatic capacity, there is a large window of opportunity for early management of this lethal disease. An ideal diagnostic method for PC should definitively distinguish malignant lesions from benign ones and detect early-stage disease and preneoplastic conditions, such as PanINs and cystic mucinous lesions with risk of progression.

There are many challenges in the early detection of PC, including its asymptomatic nature, the lack of imaging exams able to detect minimal lesions and the absence so far of sensitive and specific molecules in body fluids. Recently described circulating biomarkers associated with PC initiation and progression, easily detectable in blood, followed by confirmative diagnosis based on imaging and pathologic results might be the future ideal strategy for screening and diagnosing PC.

A number of circulating biomarkers have been widely studied, but validation for routine clinical use is still needed. The lack of sufficient samples from non-invasive precursor lesions and early-stage PC must be addressed, and animal models are important tools for research. In fact, extensive understanding of the fundamentals of PC development and the nature of precursor lesions is crucial prerequisite toward discovering and applying novel biomarkers.

Exosomes have been proving to be reliable candidates as PC biomarkers, as its contents, namely DNA, RNAs, proteins, lipids, and metabolites are largely derived from tumor cells. In this way, tracking these molecules will conduct to the knowledge of cell-type of origin, and also importantly the specificity for pre-metastatic niches formation and distant colonization.

Its potential utilization as biomarkers, besides the ultimate goal of early detection of PC, can also play an important role in monitoring disease progression.

Moreover, considering its excellent biodistribution, biocompatibility and cell-specific nature, exosomes can be used, in the future, as drug delivery vehicles.

The majority of studies addressing exosomes as biomarkers have been based on patients with established diagnosis of PC. However, and having in mind that when facing this dismal disease, the earlier the diagnosis the better the chance for cure, it would be of great interest to access the performance of these biomarkers in the context of high-risk individuals screening.

Continuous investigation is needed to ascertain the potential of these biomarkers, so that, hopefully, in the near future, they will provide the opportunity for early diagnosis, treatment intervention and increasing survival of PC patients.

AUTHOR CONTRIBUTIONS

PM-R did the bibliographic search and wrote the initial manuscript. GM and SM critically revised the manuscript and actively contributed to it.

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Conflict of Interest Statement: SM has ownership interests (patents).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.4. ORIGINAL RESEARCH ARTICLES

To further acknowledge the role of EUS and biomarkers (specially GPC1) in the management of PDAC and its precursor lesions, the present thesis is composed of 4 original research papers. These papers present and discuss data from the cross-sectional study that we conducted in a prospective cohort of patients with PDAC, controls and patients belonging to associated risk groups:

- a) A letter to the editor commenting the study “Validation of the American Gastroenterological Association (AGA) guidelines on management of intraductal papillary mucinous neoplasms: more than 5 years of follow-up” and giving insights of the preliminary data of our ongoing investigation about the potential role of exosomal GPC1 in individualizing surveillance of pancreatic cysts - **Paper 5**.

Moutinho-Ribeiro P, Melo S, Macedo G.

Glypican-1 circulating exosomes: a promising clue to individualize surveillance of pancreatic cysts?

Eur Radiol. 2018 Jul;28(7):3018-3019.
DOI: 10.1007/s00330-018-5321-6.

- b) A manuscript that reports a clinical case highlighting the role of GPC1 as a potential tool in the risk stratification for malignant transformation of pancreatic mucinous cysts, one of the most important PDAC precursor lesions - **Paper 6**.

Moutinho-Ribeiro P, Costa-Moreira P, Adem B, Batista I, Almeida M, Barroca H, Lopes J, Carneiro F, Melo SA, Macedo G.

Exosomal glypican-1 for risk stratification of pancreatic cystic lesions: A case of pathological progression in the absence of any suspicious imaging finding.

Pancreatology. 2020 Apr;20(3):571-575.
DOI: 10.1016/j.pan.2020.01.015.

- c) A manuscript that analyses the role of GPC1 as a complement to EUS in the discrimination between PDAC and CP, that can be specially demanding in the mass-forming presentation of this inflammatory condition - **Paper 7.**

Moutinho-Ribeiro P, Adem B, Batista I, Silva M, Silva S, Ruivo CF, Morais R, Peixoto A, Coelho R, Costa-Moreira P, Lopes S, Vilas-Boas F, Lopes J, Durães C, Barroca H, Carneiro F, Melo SA, Macedo G.

Exosomal glypican-1 discriminates pancreatic ductal adenocarcinoma from chronic pancreatitis.

Dig Liver Dis. 2021 Nov 25;S1590-8658(21)00842-2.

DOI: 10.1016/j.dld.2021.10.012. Epub ahead of print.

- d) A manuscript that evaluates the combination of EUS and GPC1 for the diagnosis and stratification of cancer precursor lesions (namely mucinous cystic neoplasms), as well as for screening purpose in a cohort of patients at increased risk for PDAC development (hereditary risk individuals and patients diagnosed with NLOD) - **Paper 8.**

Moutinho-Ribeiro P, Batista I, Quintas S, Adem B, Silva M, Morais R, Peixoto A, Coelho R, Costa-Moreira P, Medas R, Lopes S, Vilas-Boas F, Baptista M, Dias-da-Silva D, Esteves AL, Martins F, Lopes J, Barroca H, Carneiro F, Macedo G, Melo SA.

Exosomal glypican-1 is elevated in pancreatic cancer precursors and can signal genetic predisposition in the absence of endoscopic ultrasound abnormalities.

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DOI: <https://dx.doi.org/10.3748/wjg.v28.i31.4310>.

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PAPER 5

Moutinho-Ribeiro P, Melo S, Macedo G.

Glypican-1 circulating exosomes: a promising clue to individualize surveillance of pancreatic cysts?

Eur Radiol. 2018 Jul;28(7):3018-3019.

DOI: 10.1007/s00330-018-5321-6.



Glypican-1 circulating exosomes: a promising clue to individualize surveillance of pancreatic cysts?

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The Editor

We read with great interest the article published by Imbe et al. [1] in *European Radiology* (Jan 2018) titled ‘Validation of the American Gastroenterological Association (AGA) guidelines on management of intraductal papillary mucinous neoplasms: more than 5 years of follow-up’. In this paper, the authors discuss a ‘hot’ topic in pancreatic diseases related to the best approach for managing cystic lesions, which are being diagnosed more frequently since the increasing use of sectional imaging modalities. In this validation study, data were analysed for 392 patients with intraductal papillary mucinous neoplasms (IPMNs) and at most one high-risk feature who were periodically followed up for more than 1 year with imaging tests (group 1) and for 159 IPMN patients without worsening high-risk features after 5 years (group 2: stop surveillance group). In the first group, pancreatic cancer (PC) was identified in 12 patients (27.3 %) when endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) was indicated and none in the non-EUS-FNA indication group ($p < 0.01$). On the other hand, in the ‘stop surveillance group’, PC was identified in three patients (1.9 %) at 84, 103 and 145 months, respectively. These observations led the authors to conclude that PC risk and mortality for IPMNs not showing significant change for 5 years is likely to be low, and the non-EUS-FNA indication can provide reasonable decisions; however, three patients without worsening high-risk features for 5 years developed PC, so the ‘stop

surveillance strategy’ supported by AGA guidelines [2] should be reconsidered.

In fact, controversy exists about the best algorithm to manage these apparently low-risk lesions. Older guidelines such as the ones proposed by the International Consensus [3] and the European Consensus [4] suggest a life-long closer follow-up. On the other hand, the more recent guidelines by the American Gastroenterology Association [2] recommend discontinuation of surveillance at 5 years in the absence of significant changes.

In the same line of investigation, in a recent study published by Crippa et al. [5] in the *American Journal of Gastroenterology*, the authors support the idea that active surveillance beyond 5 years is required for presumed branch-duct intraductal papillary mucinous neoplasms (BD-IPMN) with no high-risk stigmata (HRS) or worrisome features (WF) undergoing non-operative management. In the Crippa et al. study [5], 144 patients with BD-IPMN were followed for a median of 84 months, with at least annual magnetic resonance imaging and/or magnetic resonance cholangiopancreatography, and changes during follow-up were observed in 48 % of the patients. Remarkably, new-onset WF/HRS was observed in 26 patients (18 %) after a median follow-up period of 71 and 77.5 months from diagnosis, respectively, and without previous changes in 19 (73 %) of them. Although considering the study limitations, these observations led the authors to conclude that discontinuation of surveillance of these apparently ‘inoffensive’ lesions cannot be recommended and, instead, an algorithm with intensification of follow-up is proposed after 5 years of follow-up. Nevertheless, the authors recognize that discontinuation of surveillance should be considered in patients who are unfit for surgery because of age and/or relevant comorbidities.

In the same issue of the *American Journal of Gastroenterology*, the Editorial by James Farrell [6] titled ‘Stopping pancreatic cyst surveillance’, critically discusses the pros and cons of different approaches to BD-IPMN management. In James Farrell’s opinion, sufficient data (especially

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cost-effectiveness studies) are still lacking for a proper decision for continuing or stopping surveillance of presumed low risk BD-IPMNs. This controversy needs to balance the real risk of malignancy or developing malignancy and IPMN-related mortality, with the patient's life expectancy, quality-of-life expectations and mortality from non-pancreatic-related causes. Although highlighting the occurrence of the late events during surveillance described by Crippa et al. [5], James Farrell [6] advocates that more prospectively collected information about risks of malignancy after cyst stability for 5–10 years is need. Moreover, the strategy proposed by Crippa et al. [5] raises some critical issues related to the significant costs associated with repeat imaging. In fact, the improved life expectancy and survival from non-pancreatic malignancies, in association with growing diagnosis of ever smaller BD-IPMNs, makes this issue more critical.

What seems consensual is that, in the future, the development of biomarkers dedicated to identify IPMNs with different risks of being or becoming malignant will certainly help to minimize this controversy. Several promising molecular biomarkers have been described [7]. Nevertheless, to date, besides the recognized value of carcinoembryonic antigen levels in the cyst's fluid, none of them have proved to show definitive superiority in risk stratification of pancreatic cysts.

In this field, our group is conducting a prospective study combining endoscopic ultrasound findings and characterization of a group of serum and tissue molecular biomarkers in patients diagnosed with pancreatic adenocarcinoma (PDAC) or harbouring a risk condition for it, including chronic pancreatitis, family history of PDAC or an associated hereditary syndrome, as well as a diagnosis of IPMN or other cystic mucinous neoplasms (CMNs).

The molecular markers we have been studying include a panel of microRNAs and, most importantly, the recently described glypican-1 (GPC1), a cell surface proteoglycan, overexpressed in PDAC and specifically enriched on cancer-cell-derived circulating exosomes (crExos) [8]. Melo et al. [8] demonstrated that levels of GPC1+ crExos correlated with tumour burden and the survival of patients with PDAC. Interestingly, in our ongoing study, preliminary data indicate that levels of GPC1+ crExos are significantly higher in patients with PDAC as well as in some patients with CMNs, when compared to other associated group risks. In fact, in our cohort of CMNs (diagnosed with clinical, biochemical and echoendoscopic criteria), data on serological biomarkers seem to be categorized into two possible distinct groups of patients: those with GPC1+ crExos levels as high as PDAC patients and those with levels similar to control groups. In the near future we will know if this is truly a clue for stratifying

pancreatic mucinous cysts according to their malignant potential and enable us to perform an individualized management approach.

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Compliance with ethical standards

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Statistics and biometry No complex statistical methods were necessary for this paper.

Informed consent Written informed consent was obtained from all subjects (patients) in this study.

Ethical approval Institutional Review Board approval was obtained.

Methodology

• performed at one institution

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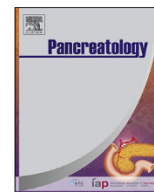
PAPER 6

Moutinho-Ribeiro P, Costa-Moreira P, Adem B, Batista I, Almeida M, Barroca H, Lopes J, Carneiro F, Melo SA, Macedo G.

Exosomal glypican-1 for risk stratification of pancreatic cystic lesions: A case of pathological progression in the absence of any suspicious imaging finding.

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Letter to the Editor

Exosomal glypican-1 for risk stratification of pancreatic cystic lesions: A case of pathological progression in the absence of any suspicious imaging finding

A B S T R A C T

Keywords:

Glypican-1
Pancreatic cystic lesions
Pancreatic cancer
Cancer screening
Neoplastic biomarkers

The clinical management of patients with pancreatic cystic lesions is of utmost importance to identify those at high risk for pathological progression. Current recommendations are guided by clinical presentation and radiologic criteria, but the results fall short for a disease that the only curative option is surgical resection. There is an urgent need for the introduction of biomarkers that can help in risk assessment of such lesions.

We report a case of a pancreatic cystic lesion without imagiological findings suggestive of advanced disease, and high levels of a circulating biomarker, glypican-1 (GPC-1), which parallel those of patients with pancreatic cancer. One year after, the patient revealed malignant progression at follow-up. Our report is unprecedented in the literature. It describes a clinical case in which a biomarker was positive for a patient that only showed progression one year after its detection. This clinical information goes beyond the current knowledge in the field because it shows that the introduction of liquid biopsy and biomarkers is a highly promising clinical tool for the non-invasive assessment of pancreatic cancer precursor lesions, ultimately increasing the rate of patients eligible for surgical resection.

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The diagnosis of pancreatic ductal adenocarcinoma (PDAC) at early stages, suitable for curative resection, is not frequent, occurring in no more than 20% of cases, as the disease usually develops silently, with few or no symptoms. Pancreatic cancers detected incidentally, the so called incidentalomas, appear to be associated with improved survival, supporting that screening might be a strategy to improve overall outcomes [1,2]. Many programs have been tested but, so far, none of them has recognized cost-effectiveness.

Optimal screening strategy should focus on identification of high-grade non-invasive precursor neoplastic lesions, amenable for curative surgical resection [3,4]. Mucinous cystic lesions are among them, but their identification and stratification according to potential malignant behaviour is still extremely difficult [3,4]. Highly sensitive and specific biomarkers, alone or in combination with imaging techniques, should be considered to improve effectiveness of upcoming screening strategies [5].

As of now, in clinical practice no biomarker has been proved satisfactorily accurate for routine use in PDAC screening or precursor lesions' risk assessment [5].

We report a case of a patient with a pancreatic cystic lesion without any imagiological findings suggestive of advanced disease - but presenting high levels of a newly recognized circulating biomarker, glypican-1 (GPC-1) [6]- that revealed malignant progression at follow-up.

A 58-year-old woman with past history of breast cancer in remission for 4 years was referred for the evaluation of a pancreatic cystic lesion, incidentally discovered in follow-up imaging studies.

Endoscopic ultrasound (EUS) showed a 16 mm anechoic regular cystic lesion in the pancreatic body, communicating with the main pancreatic duct (MPD), suggesting a branch-duct intraductal papillary mucinous neoplasia (BD-IPMN). EUS did not reveal any clinical suspicious findings, such as: irregular septa, mural nodules or dilation of the MPD (Fig. 1A and B). Despite the absence of worrisome features or high-risk stigmata, according to the latest International Consensus Guidelines [7], but given the patient's anxiety caused by the presence of a new potential neoplastic lesion, we decided to perform fine-needle aspiration (FNA) for biochemical and cytological characterization of cystic fluid. Biochemistry was compatible with a mucinous origin (CEA – 662 ng/mL; amylase – 252 U/L), and cytology showed epithelial mucinous cells with low grade atypia (Papanicolaou Classification IVb. Neoplasm, other) [8] (Fig. 2A).

A follow-up EUS-FNA was performed one year later, showing the persistence of the cystic lesion, with stable dimensions and no worrisome features. At this time, cytology showed a diagnosis of mucinous cyst with high grade atypia, based on the presence of tridimensional epithelial groups with complex and disorganized architecture (overcrowding, loss of polarity) composed of cylindric epithelial mucosecreting cells, with irregular large nucleus with membrane enhancement and nucleolus, along with mitotic figures (Papanicolaou Classification VI- malignant) (Fig. 2B).

After multidisciplinary discussion it was decided to perform a distal pancreatectomy with the patient agreement. The histological evaluation of the surgical specimen revealed a high-grade IPMN

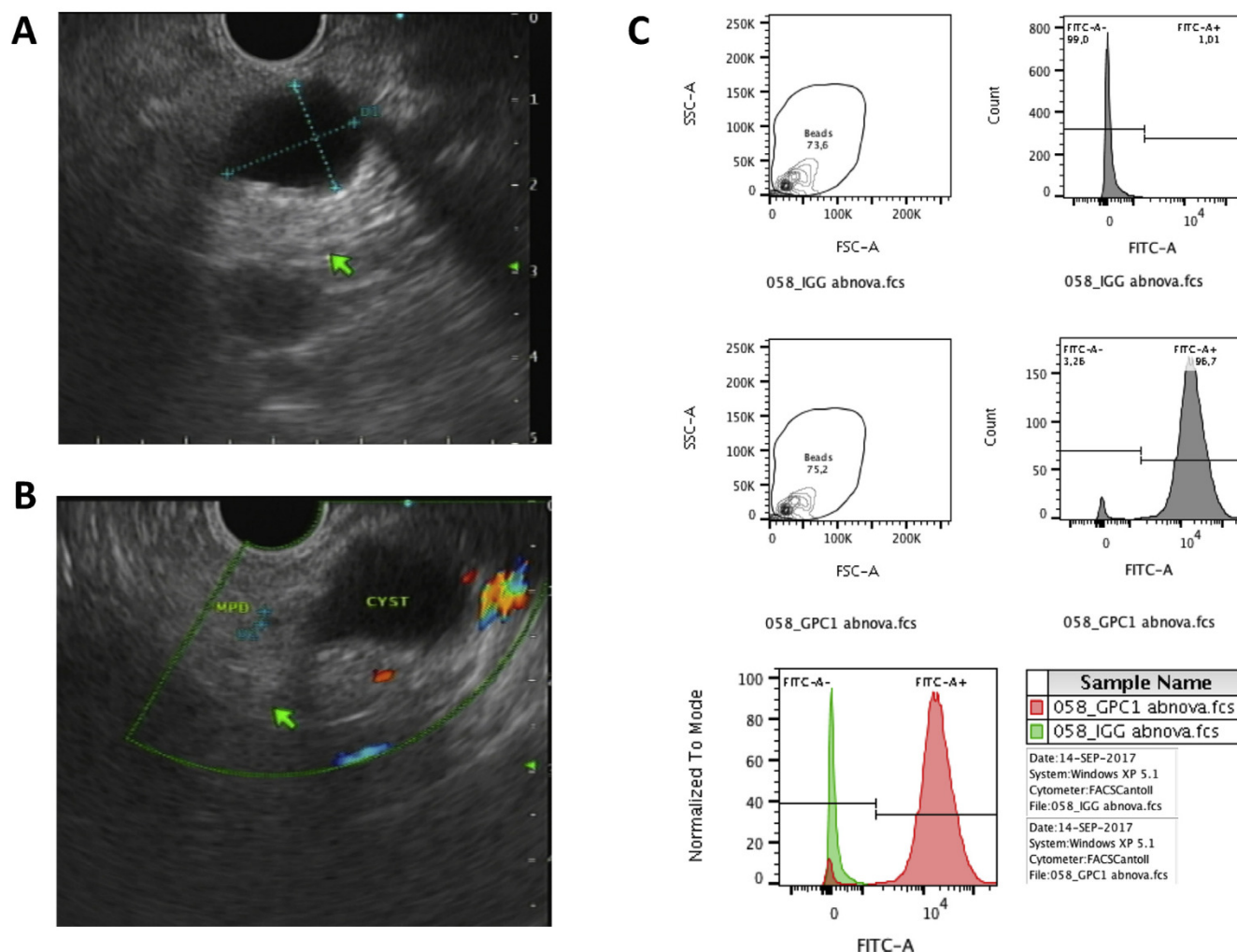


Fig. 1. A) Endoscopic ultrasound (EUS) showing a 15.7x12.8mm anechoic regular cystic lesion, located in the pancreatic body (arrow). B) Color doppler sign in the splenic vein but not in the cystic lesion (CYST); the main pancreatic duct (MPD) had a normal caliber of 1.4mm (arrow). C) Flow cytometry analyses for the % of GPC1+ circulating exosomes on beads. Upper panels represent negative control (secondary only) and lower panels represent anti-GPC1 analysis using the exact same gate strategy. Overlay of control (green) and GPC1 (red) are represented on the graph on the bottom with the time stamp for the raw data acquisition.

with biliopancreatic phenotype (Fig. 3). The remaining parenchyma showed scattered lesions of Pancreatic Intraepithelial Neoplasia of low grade (PanIN I and II subtypes).

At the time the first EUS was performed, our group was conducting a prospective study aiming to evaluate EUS findings along with the assessment of exosomal GPC-1 in patients with increased risk for PDAC development. This specific patient, harbouring a mucinous cystic lesion, was included in the cohort. Interestingly, the percentage of circulating exosomes positive for GPC-1 was elevated (96.7%, Fig. 1C), comparable to those seen in PDAC patients [6].

Pancreatic cysts are lesions commonly found in clinical practice, especially after the disseminated use of cross-sectional imaging diagnostic techniques. Although the majority of these lesions are benign, there is a risk of 15% that these lesions represent precursor lesions of invasive carcinoma, thus requiring further monitoring [9]. Surgical resection is the only curative option, but is associated with considerable morbidity and mortality, therefore, the accurate selection of patients is essential. [10].

Histologically, IPMNs are characterized by epithelial papillary growths within the ductal system, usually associated with secretion of thick mucin. Considering the site and extent of involvement,

three types of IPMNs are recognized: MD-IPMN (main duct intra-ductal papillary mucinous neoplasm), BD-IPMN and mixed type. Four pathologic subtypes, with variable degrees of potential malignant behaviour have been described, including the gastric, intestinal, pancreatobiliary, and oncocytic ones [11,12]. The latter has been recently retired from the IPMN group, to be classified as Intraductal Oncocytic Papillary Neoplasm (IOPN) by the new WHO classification of Digestive Tumours [13]. MD- and mixed types IPMNs harbour a significant risk of malignant progression, thus surgical resection is advisable in every suitable patient [7].

Controversy exists about the appropriate management of BD-IPMNs, as they can represent a huge range of entities with different clinic-pathological relevance. In fact, by definition (World Health Organization), it is implicated that at least low-grade dysplasia is present in all BD-IPMNs, but they can present the complete spectrum of changes, including high-grade atypia and invasive cancer [13,14].

The accurate diagnosis of these advanced cytologic lesions in the setting of BD-IPMN investigation represents a field where critical efforts should be made, aiming the detection of PDAC in its early stages.

The accuracy of computed tomography (CT) and magnetic

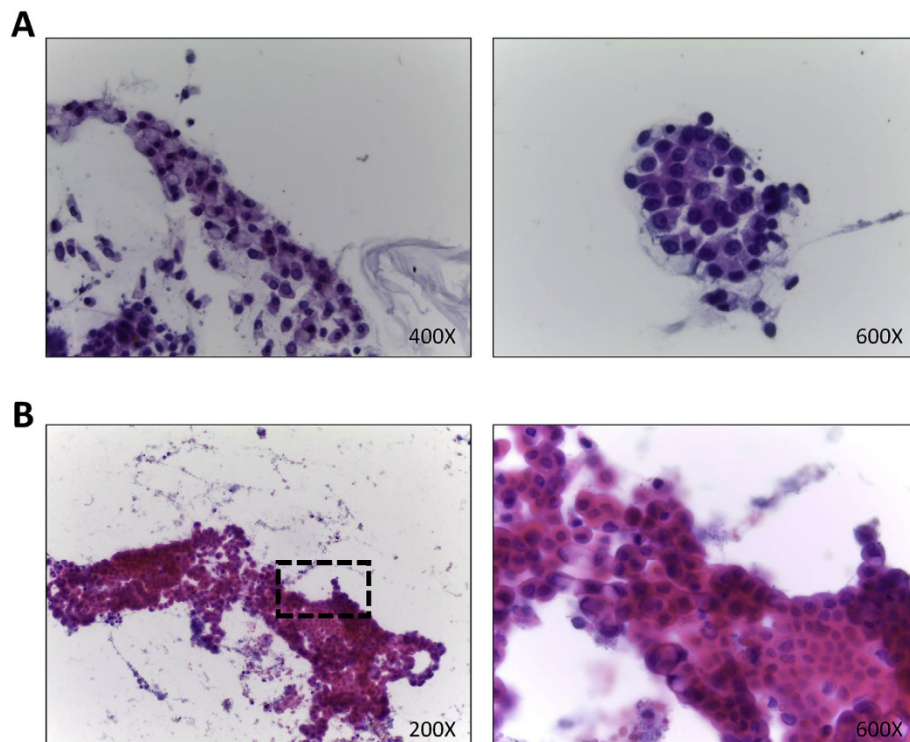


Fig. 2. A) Cytology corresponding to the first EUS-FNA, revealing epithelial mucinous cells with low grade atypia. B) Cytology corresponding to the second EUS-FNA, revealing epithelial mucinous cells with high grade atypia.

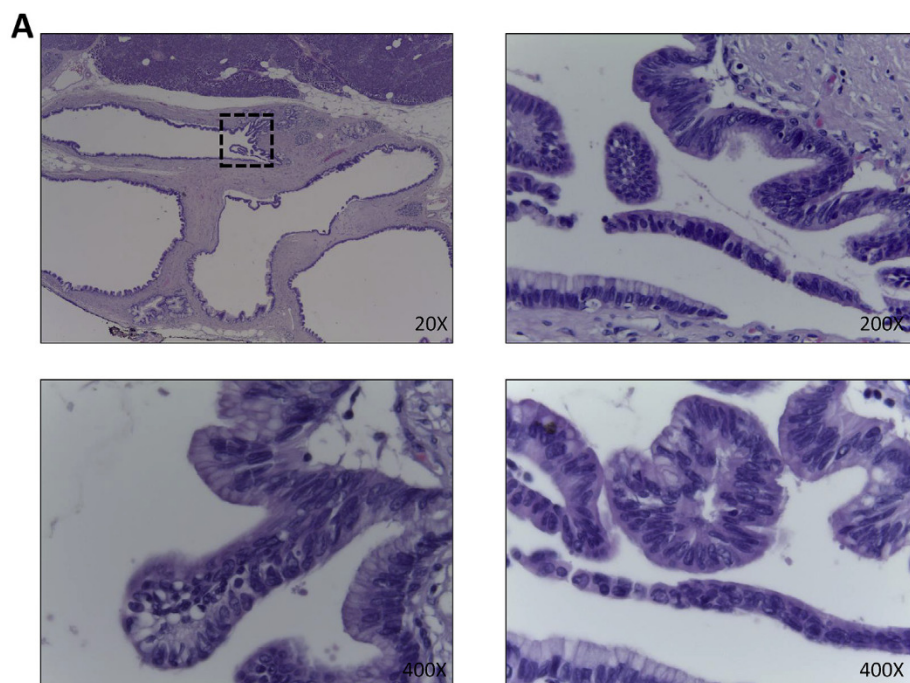


Fig. 3. Histology of the surgical resected specimen. Remark in the selected rectangular area, the presence of high-grade focal lesions, represented in greater magnification, in the three adjacent images.

resonance imaging (MRI) remains limited. EUS has higher resolution and is very useful in cases where a diagnosis of BD-IPMN is uncertain or where worrisome features have been detected by CT/MRI, and, ultimately, for assessing malignancy in high-risk

individuals [15,16].

Current practice guidelines focus on clinical parameters and imaging findings, in order to predict signs of an advanced disease, and consequently refer it to further evaluation using EUS (with or

without FNA) and eventually surgical resection [7,17,18].

EUS-FNA is considered a safe procedure, with mortality and morbidity rates less than 0.05% and 5%, respectively [19]. Nevertheless, due to the relatively low cellular content of cyst fluid, the yield of cytology is not good enough in providing an accurate diagnosis of mucinous neoplasms and exclude malignancy, with a disappointingly low sensitivity around 50% [20].

Current management guidelines represent important tools to be used to broadly stratify patients with pancreatic cystic lesions in risk groups and as a platform to streamline physicians dialogue with their patients. Importantly, despite several pitfalls, all management guidelines highlight the need of identification of worrisome features or high-risk stigmata representing precancerous or even malignant lesions, such as presence of solid mural nodules, main pancreatic duct dilation, increased cystic size, and high-grade cytologic atypia in FNA samples. Advances in tissue sampling of cystic wall and cyst fluid analysis will help the identification of patients that could benefit most from surgical resection [14].

Nevertheless, taking in account the limitations of the available imaging techniques, the clinical management of patients with pancreatic cystic lesions in order to identify those at high risk for pathological progression, that could therefore be eligible for a surgical resection (the only curative option for invasive carcinoma), is still imperfect.

Concurrently, the only available serum biomarker for clinical use in pancreatic diseases, CA 19-9, albeit having a role in identifying preoperatively the malignant nature of some mucinous cystic lesions [21], have never shown the ability to predict the potential for pathological progression of apparently inoffensive ones. Therefore, there is an urgent need for the introduction of newly biomarkers that can help in the risk assessment of cystic lesions before malignant transformation occurs. In the last years, evolving research in this field, including the liquid biopsy, have shown promise. Indeed, GPC-1 circulating exosomes have been shown to detect early stages of PDAC [6]. Exosomes are secreted by all cells, circulate in many body fluids including the blood, and constitute lipid-bilayer-enclosed extracellular vesicles, containing proteins and nucleic acids [22].

In our patient, by the time of initial EUS exam, a high percentage (96.7%) of GPC-1 circulating exosomes represented a very surprising result, considering the diagnosis of an apparently innocent pancreatic cystic lesion. In this specific case, despite the absence of suspicious clinical or imagiological findings, we decided to perform FNA given the anxiety of the patient caused by the past history of breast cancer and the uncertainty of pancreatic lesion aetiology. In another clinical setting, this patient might not have been referred for EUS and probably would be advised to follow a surveillance program with CT or MRI. However, one year after the first diagnosis, the cytological analysis of the cyst fluid collected during the second EUS-FNA revealed the presence of high-grade atypia, which was confirmed by two experienced cytopathologists. In the subsequent surgically resected specimen, the final diagnosis of an IPMN with biliopancreatic phenotype and high-grade atypia was made. Scattered low grade PanIN lesions were present in the adjacent pancreatic parenchyma.

Taking into consideration the clinical, pathological and molecular data collected, we believe there is sufficient grounds to establish that the observed high values of GPC-1 positive circulating exosomes (blindly analysed one year before final diagnosis) possibly correlated to the intrinsic high potential for cytological progression that lastly occurred in this apparently “inoffensive” pancreatic cystic lesion. Our case report is unprecedented in the literature, as it describes a clinical case in which a biomarker (GPC-1) showed to be positive for a patient that only showed disease progression one year after the biomarker detection.

Previously, we also reported [23] that in our prospective cohort of cystic mucinous lesions, two subsets of patients appeared to exist: those with levels of circulating exosomal GPC-1 as low as controls and benign pancreatic conditions, and those with higher levels, some in the range described in cancer patient samples. Future studies will clarify the real meaning of these observations.

In agreement with our finding, a recently published study [24] showed that combination of three diagnostic tools, namely GPC-1, CA 19-9, and EUS-FNA, increased the diagnostic performance of CA 19-9 alone (sensitivity and specificity as low as 70% and 68%, respectively) and displayed a diagnosis accuracy of 84% for cancer. These authors also stated that CA 19-9, the only biomarker currently used in clinical practice, was not a useful prognostic marker for early stages, showing a very low sensitivity of 37% and a specificity of 87%.

In our patient, the finding that a specific biomarker, GPC-1, had the capacity to assess the high risk of an otherwise unsuspected cystic lesion, before malignant transformation occurs, may represent a window of opportunity for future management of pancreatic cystic mucinous lesions. In this way, we do think that our study expands the current knowledge in the field of pancreatic cancer therapeutics and supports the concept that liquid biopsy is a highly promising clinical tool for the non-invasive diagnosis and assessment of pancreatic cancer precursor lesions, ultimately increasing the rate of patients eligible for surgical resection. In our opinion, the present observation highlights the emergent need to incorporate biomarkers in the upcoming clinical guidelines addressing the management of cystic lesions, which, as mentioned before, are currently guided only by clinical presentation and radiologic features. In fact, tacking in account the present limitations of these criteria, we believe that this observation can be of great significance for those who, on everyday clinical practice, have to decide what is the best strategy to manage patients like this one.

Hopefully, one might postulate that, sooner than expected, we will be able to improve the dismal prognosis associated with pancreatic cancer, by means of its early diagnosis and treatment.

Informed consent

The authors confirm that informed consent was obtained for the publication of this case.

Declaration of competing interest

S. A. M. has ownership interests (patents). All authors disclosed no other potential conflicts of interest or financial relationships relevant to this publication.

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3.4.3.

PAPER 7

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Liver, Pancreas and Biliary Tract

Exosomal glypican-1 discriminates pancreatic ductal adenocarcinoma from chronic pancreatitis

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ABSTRACT

Background and aims: Pancreatic ductal adenocarcinoma (PDAC) diagnosis can be difficult in a chronic pancreatitis (CP) background, especially in its mass forming presentation. We aimed to assess the accuracy of glypican-1-positive circulating exosomes (GPC1⁺crExos) to distinguish PDAC from CP versus the state-of-the-art CA 19-9 biomarker.

Methods: This was a unicentric prospective cohort. Endoscopic ultrasound with fine-needle aspiration or biopsy and blood tests (GPC1⁺crExos and serum CA 19-9) were performed.

Results: The cohort comprised 60 PDAC and 29 CP (7 of which mass forming - MF) patients. Median levels of GPC1⁺crExos were significantly higher in PDAC (99.7%) versus CP (28.4%; $p < 0.0001$) with an AUROC of 0.96 with 98.3% sensitivity and 86.2% specificity for a cut-off of 45.0% ($p < 0.0001$); this outperforms CA 19-9 AUROC of 0.82 with 78.3% sensitivity and 65.5% specificity at a cut-off of 37 U/mL ($p < 0.0001$). The superiority of GPC1⁺crExos over CA 19-9 in differentiating PDAC from CP was observed in both early (stage I) and advanced tumors (stages II-IV).

Conclusion: Levels of GPC1⁺crExos coupled to beads enable differential diagnosis between PDAC and CP including its mass-forming presentation.

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is most often diagnosed at an advanced stage with a poor overall 5-year survival rate of ~9% [1]. To improve prognosis of PDAC, it is crucial to identify biomarkers that allow an accurate diagnosis at early stages. In patients with chronic pancreatitis (CP) it may be difficult to exclude the presence of neoplastic changes even after performing tissue acquisition guided by endoscopic ultrasound (EUS). Therefore, a biomarker that better discriminates PDAC from CP—especially in its demanding mass-forming (MF) presentation—would constitute a major improvement. The current gold-standard biomarker used in the clinic is carbohydrate antigen 19-9 (CA 19-9), but this has limitations in early detection [2]. Recent advances have shown

that the membrane-anchored proteoglycan glypican-1 (GPC1) is a good candidate biomarker for early detection of PDAC [3–10]. Distinct studies showed that GPC1 is overexpressed in circulating exosomes (crExos) of patients with PDAC versus healthy subjects [3–6,8–11]. Even though this same finding was described when comparing PDAC with CP, no study to date, has investigated these aspects on MF CP. We evaluated the accuracy of GPC1⁺crExos to discriminate between CP and PDAC using a comprehensive cohort of PDAC and CP patients and compared the performance of GPC1⁺crExos with that of CA 19-9 serum levels for malignancy detection in CP MF and non-mass-forming (non-MF) types.

2. Materials and methods

2.1. Study design

We conducted a cross-sectional study with a prospective unicentric cohort including PDAC and CP patients. All patients gave

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informed consent and were submitted to blood sample collection at the time of EUS. The study was approved by the Ethical Committee of Centro Hospitalar Universitário de São João (CHUSJ), Porto, Portugal. The cohort inclusion period was between 10/2016 and 01/2019.

PDAC patients were included when a confirmed diagnosis by cytology and/or histology was obtained after EUS FNA (fine-needle aspiration) and/or FNB (fine-needle biopsy) [12]. The diagnosis of CP was defined after clinical and cross-sectional imaging evidence of the disease (CT and/or MRI) and at least 5 criteria of the International Working Group / Rosemont Consensus Definitions [13–17] for CP in the EUS examination. In addition, laboratory analysis was performed on samples from all patients: biochemistry analysis from blood and feces samples to assess exocrine pancreatic insufficiency. MF CP cases were assessed with FNA/B (in five patients more than one procedure (minimum 2, maximum 4) with EUS-guided tissue acquisition). The final diagnosis of CP was assumed only after a follow-up period of at least 24 months given the difficulty of excluding malignant transformation particularly in its MF presentation.

The exclusion criteria were patients unable or unwilling to give their informed consent, individuals younger than 18 years of age, pregnancy or breast-feeding, contraindications to endoscopic procedures or contrast administration, contraindications to CT or MRI, coagulopathy (prothrombin time >50% of control, aPTT >50 s, or INR >1.5), patients on chronic anticoagulation, platelet count <50,000 per microliter, and inability to tolerate sedated upper endoscopy due to cardio-pulmonary instability or other contraindication to endoscopic procedures.

All subjects were observed, examined, and followed-up in our Department of Gastroenterology of Centro Hospitalar Universitário de São João, Porto, Portugal. A complete personal and family clinical history was registered. At the time of subjects' inclusion, a blood sample was collected for hemogram as well as renal, liver and pancreatic chemistry at the CHUSJ laboratory. A separate blood sample collected at the same time was used for analysis of serum CA 19–9 and GPC1 on crExos.

Demographic data was recorded and included sex, age, previous history of pancreatic disease, smoking and drinking habits, previous pancreatic surgery, diabetes, body mass index, symptoms (abdominal pain, nausea, weight loss, anorexia, jaundice), laboratory values, and previous results of cross-sectional images (CT, MRI or MRPC). Data of EUS evaluation at the time of inclusion in the study as well as transechoendoscopic or surgically collected specimens were also recorded.

2.2. EUS examination

All patients were submitted to EUS performed by three experienced endoscopists under sedation (propofol or pethidine plus midazolam as assisted by an anesthesiologist). Linear scopes from Olympus® (GT-UCT140, GT-UCT180) and Pentax® (EG-3670UTK, EG-3870UTK) along with Olympus® EU-ME2 and Hitachi Avius® image processors were used.

EUS-FNA/B was performed in the presence of suspicious solid or indeterminate lesions. During the EUS-FNA/B procedure, 25/22/19 G FNA and 22 G FNB needles (Wilson-Cook® and Boston Scientific®) were used. The most commonly used needle was the 22 G FNA, and a minimum of three passes were performed when not using ROSE (Rapid OnSite Evaluation). Suction was applied as a rule. A change of the needle caliber/type was considered if the macroscopic examination of the specimen seemed unsatisfactory or if dictated by ROSE information. Masses located at the head of the pancreas were accessed through the duodenum while those at the body or tail were accessed through the stomach. Doppler and elastographic assistance helped select the safest needle path and

best site for puncture. Contrast-enhanced EUS (CEUS) image acquisition was done in selected cases using the SONOVUE® (microbubbles) contrast. Adverse events of EUS or related procedures were recorded.

2.3. Pathological examination

The microscopic evaluation of EUS-FNA/B samples was done according to the Papanicolaou Society of Cytopathology System for Reporting Pancreaticobiliary Cytology [12]. According to these criteria, there is no established set number for cellular adequacy in pancreaticobiliary cytology and the amount of sample regarded as being diagnostic is influenced by the pathologist experience and expertise in interpreting pancreaticobiliary cytology specimens. All microscopic examinations were analyzed by experienced pathologists in this area and contextualized relative to the patients' clinical history and imaging data. Hence, samples were considered non-diagnostic if they did not meet the criteria of the Papanicolaou Society of Cytopathology System for Reporting Pancreaticobiliary Cytology considering the patients' history. In the case of an inconclusive pathological report, a second EUS FNA/B was preferably performed with ROSE.

2.4. Human blood samples

Blood samples were collected for every patient at the time of the EUS examination and centrifuged at 2000 g for 10 min at 4 °C to isolate serum. Serum samples were stored at –80 °C until analyzed.

2.5. Isolation of exosomes from human serum samples

Serum samples were thawed on ice and 200 µl was spun at 10,000 rpm for 2 min. The supernatant was recovered and 200 µl NaCl was added. Samples were filtered through a 0.2-µm pore filter (Whatman), and 11 mL of NaCl were added for ultracentrifugation at 100,000 g overnight at 4 °C. The supernatant was discarded, and pelleted exosomes were resuspended in 300 µl of 1X PBS and used for flow cytometry analysis. Ten microliters of the 300 µl exosomes sample were used for nanoparticle tracking analysis (NanoSight NS300) after a 1:100 dilution in 1X PBS. Particle size and concentration were determined based on this analysis.

2.6. GPC1 analysis of exosomes bound to beads by flow cytometry

Upon determination of the concentration of particles by nanoparticle tracking analysis, the same exact number of exosomes for all patients (PDAC and CP) were used for flow cytometry analysis. Exosomes were attached to 4 µm aldehyde/sulfate latex beads (Thermo Fisher A37304). Five microliters of beads were resuspended in 100 µl of 1X PBS and incubated for 15 min at room temperature with continuous rotation. The exosome sample was added at a concentration of 3.0×10^9 , and the volume adjusted with 1X PBS up to 400 µl. Samples were incubated at room temperature with continuous rotation for 15 min followed by 30 min incubation at 4 °C. An equal volume (i.e., 400 µl) of glycine 1 M in 1X PBS was added and allowed to rotate at 1 h at room temperature. Samples were centrifuged for 2 min at 12,000 rpm, blocked with 10% BSA with rotation at room temperature for 45 min, centrifuged for 2 min at 12,000 rpm, and incubated with anti-GPC1 (Abnova, MAB8351, 1:240 dilution in 240 µl of 2%BSA/1XPBS) overnight with slow rotation at 4 °C. Beads were centrifuged for 2 min at 12,000 rpm, and the supernatant was discarded; beads were washed twice in 1XPBS/2% BSA, and centrifuged for 2 min at 12,000 rpm. Alexa-488-tagged secondary antibodies (Thermo Fisher Scientific, anti-mouse A-21,202) were used for 30 min at

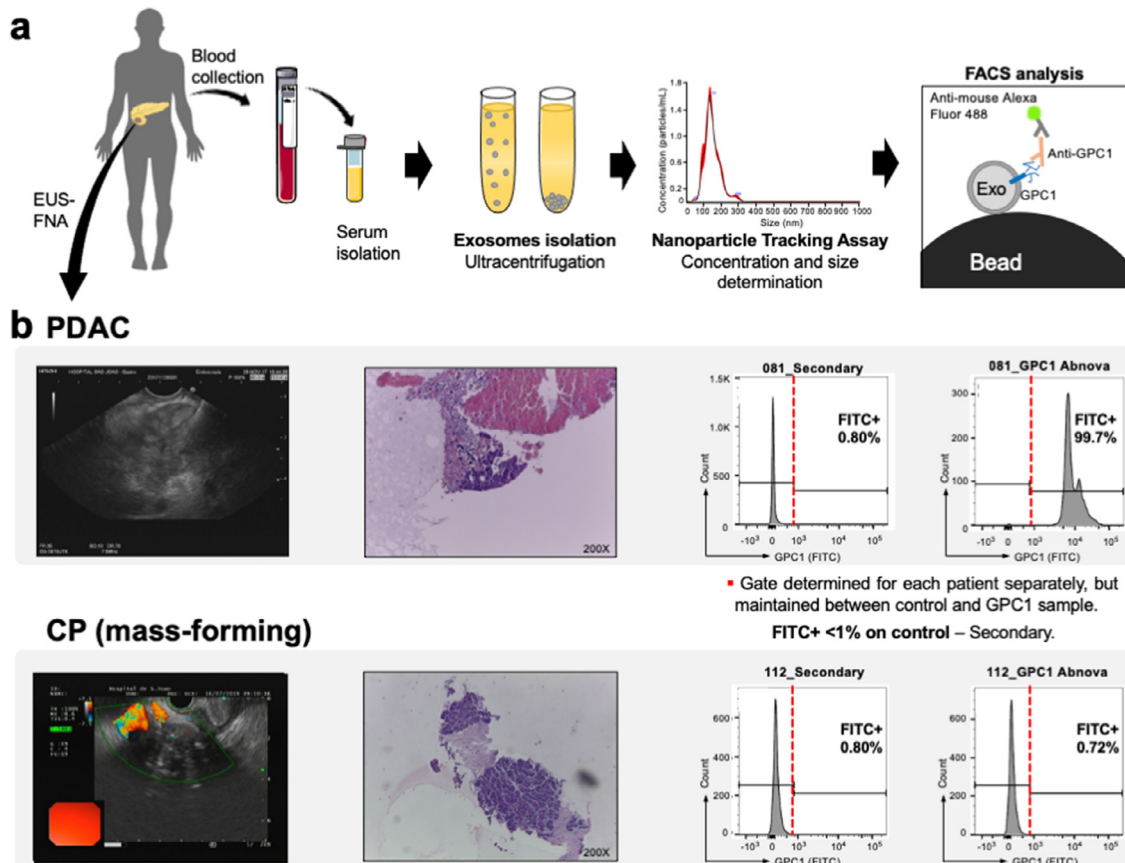


Fig. 1. Schematic representation of the study outline. **a.** Workflow starting from sample collection to data analysis. Blood samples were collected at time of endoscopic ultrasound fine-needle aspiration (EUS-FNA) performance. **b.** Representative images of EUS findings (left panel), cytology upon EUS-FNA performance (middle panel), and flow cytometry data analysis (right panel) from a patient with PDAC or MF CP.

room temperature with rotation. After the incubation with the secondary antibody, beads were centrifuged for 2 min at 12,000 rpm, the supernatant was discarded, and the beads were washed twice in 2%BSA/1XPBS and centrifuged for 2 min at 12,000 rpm. The final bead-exosome-antibody complexes were resuspended in 300 μ l of 2%BSA/1XPBS and analyzed on FACS Canto II (BD Bioscience). Secondary antibody incubation in each sample served as a control and was used to gate the beads bound with GPC1+crExos. The percentage of positive beads was determined for each patient separately by adjusting the FITC voltage when needed while always maintaining the same gate and FITC voltage between the control (secondary only always <1%) and GPC1 sample.

2.7. CA19-9 and GPC1 ELISA

Serum CA 19-9 and GPC1 protein on crExos were determined using the CA 19-9 Human ELISA Kit (Abcam, ab108642) and the GPC1 Human ELISA kit (Cloud-Clone Corp, SEB032Hu), respectively, according to the manufacturer's instructions. For CA 19-9 the cut-off of 37 U/mL was used according to the routine clinical practice of our Hospital.

2.8. Statistics

Categorical variables are described through absolute and relative frequencies and continuous variables as mean and standard deviation, median, percentiles, minimum, and maximum. Hypotheses were tested regarding the distribution of continuous variables via an independent sample *t*-test/one-way ANOVA or nonparamet-

ric Mann-Whitney and Kruskal-Wallis test depending on normal or non-normal distribution, respectively, and considering the nature of the hypothesis. The chi-squared and ANOVA tests were used for categorical variables analysis. Pearson's correlation coefficient was used to assess the statistical relationship/association between two continuous variables.

The diagnostic accuracy of GPC1+crExos and of CA 19-9 was assessed by receiver operating characteristic curves (ROC) considering 95% confidence intervals (CIs). Areas under the ROC curves (AUROC) were calculated using the SPSS software. Outliers were identified using Grubbs's test (GraphPad Prism 7.00). All graphs were created using GraphPad Prism 7.00.

3. Results

A workflow of the study with representative images of EUS, cytology, and analysis of GPC1+crExos coupled to beads in CP and PDAC samples can be found in Fig. 1. The specificity of the GPC1 recognition by the antibody was demonstrated through a sample titration experiment (Supplementary Figure 1a). Baseline demographic and clinical characteristics of all 89 patients included in the study (29 CP, 60 PDAC) are shown in Table 1. The male:female ratio was higher in the CP group (3.1:1) than the PDAC group (1.2:1; $p = 0.046$). The mean age of CP patients was significantly lower than the PDAC patients (54.0 ± 10.3 vs. 67.0 ± 11.4 , $p < 0.001$). The majority of tumors were located in the head of the pancreas and most were diagnosed in late stage—10% were amenable to surgical resection.

Table 1Baseline demographic and clinical characteristics of the study population ($n = 89$).

Cohort characteristics	CP (n/%)	PDAC (n/%)
Sex		
Male	22 (75.9)	33 (55.0)
Female	7 (24.1)	27 (45.0)
Age, years (mean$\pm$SD)	54.0 $\pm$ 10.3	67.0 $\pm$ 11.4
Mass localization		
Head	6 (85.7)	35 (58.3)
Body	1 (14.3)	21 (35.0)
Tail	0	4 (6.7)
Staging		
IA	n/a	2 (3.3)
IB	n/a	13 (21.7)
IIA	n/a	3 (5.0)
IIB	n/a	1 (1.7)
III	n/a	23 (38.3)
IV	n/a	18 (30.0)
Mass-forming (yes/no)	7 (24.1) / 22 (75.9)	n/a
EUS FNA/FNB (yes/no)	7 (24.1) / 22 (75.9)	60 (100) / 0 (0.0)
EUS FNA/FNB Results		
Malign	0	60 / (100.0)
Benign	4 (57.1)	0
Inconclusive	3 (42.9)	0
Resectability (yes/no)	n/a	6 (10.0) / 54 (90.0)

CP – Chronic Pancreatitis; PDAC – Pancreatic Ductal Adenocarcinoma; SD – Standard Deviation.

EUS FNA/FNB – Endoscopic ultrasound fine needle aspiration or fine needle biopsy; n/a – not applicable.

In the CP group ($n = 29$), seven (24.1%) patients had MF presentation in EUS and were submitted to fine-needle aspiration (FNA) and/or biopsy (FNB) to exclude malignant transformation. The cytohistological examination was conclusive in 57.1% of cases even when the biopsy was repeated with rapid onsite evaluation.

Median levels of GPC1+crExos coupled to beads were 28.4% (minimum 0.6%, maximum 99.9%; IQR:2.4–38.7%) and 99.7% (minimum 23.8%, maximum 100%; IQR:98.4–99.9%) in CP and PDAC groups, respectively ($p < 0.0001$) (Fig. 2a). No differences between groups were obtained when the levels of GPC1+crExos were determined using a commercially available ELISA kit (2.2% vs. 2.3%, $p = 0.161$) (Supplementary Figure 1b). Median levels of CA 19–9 (U/mL) were also significantly higher in patients with PDAC (311.1; minimum 6.3, maximum 818.5; IQR:57.8–575.4) compared to CP (23.4; minimum 7.1, maximum 518.8; IQR: 13.2–53.8) ($p < 0.0001$) (Fig. 2b). The median concentration of crExos was similar in both groups (Supplementary Figure 1c). The median size of crExos was significantly higher in CP patients (108.0 nm, IQR:86.8–127.0) than PDAC ones (94.1 nm, IQR:82.4–11.5) ($p = 0.047$) (Supplementary Figure 1d). However, according to the ROC curve, neither the concentration nor the size of crExos could efficiently discriminate between PDAC and CP (Supplementary Figure 1e).

In CP patients, the levels of GPC1+crExos coupled to beads and CA 19–9 were not significantly different between the subgroups of MF or non-MF cases (Fig. 2c–d). For a cut-off of 45.0%, the levels of GPC1+crExos coupled to beads presented an area under the receiver operating characteristics (AUROC) value of 0.961 (sensitivity 98.3% and specificity 86.2%; $p < 0.0001$) (Fig. 2e). Considering the levels of GPC1+crExos coupled to beads, two statistical outliers were identified: One in the PDAC group had a lower percentage (23.8%) than the mean of the group (99.7%). The other was in the CP group and presented a higher level (99.9%) than the median of the CP (28.4%). We found no malignant transformation during the surveillance of this CP patient. Nonetheless, during the 4th year of follow-up, a *de novo* 20-mm cystic lesion was detected by CT scan, and EUS-FNA was performed. The analysis was consistent with a side-branch intraductal papillary mucinous neoplasm (SB-IPMN) (amylase 12,973 U/L, CEA 382 ng/mL, hypocellular cytology, with

no epithelial cells). It had not worrisome features but deserved further close monitoring.

In comparison, and considering the entire cohort, at a cut-off of 37 U/mL (the standard clinical cut-off), the levels of CA 19–9 presented an AUROC value of 0.822 (sensitivity 78.3% and specificity 65.5%; $p < 0.0001$) (Fig. 2e).

Interestingly, one of the PDAC cases was initially recruited as a MF CP. However, upon the initial EUS, the biopsy indicated malignant transformation. Remarkably, the level of GPC1+crExos coupled to beads was high (100.0%).

We also investigated the performance of both biomarkers according to the stage of the tumor. We analyzed the levels of GPC1+crExos coupled to beads and CA 19–9 considering PDAC stages grouped in stage I ($n = 15$) vs. stages II–IV ($n = 45$). The median levels of GPC1+crExos was 99.7% in stage I (minimum 92.3%, maximum 100%; IQR:98.5–99.9%), and 99.6% in stages II–IV (minimum 23.8%, maximum 100%; IQR:98.2–99.9%; $p = 0.655$); the median serum levels of CA 19–9 were 289.3 U/mL in stage I (minimum 16.1, maximum 813.7; IQR:221.1–399.9), and 371.8 U/mL in stages II–IV (minimum 6.3, maximum 818.5; IQR:46.8–578.3; $p = 0.714$).

The AUROC values for GPC1+crExos coupled to beads in differentiating PDAC from CP were 0.974 for stage I and 0.957 for stages II–IV tumors (Fig. 3a). CA 19–9 serum levels had AUROC values in differentiating PDAC from CP: 0.861 for stage I and of 0.809 for stages II–IV (Fig. 3b).

4. Discussion

The diagnosis of PDAC in a scenario of chronic inflammatory background is often difficult because the available sectional imaging modalities lack specificity to differentiate pancreatic cancer from MF CP. Moreover, the accuracy of EUS-guided tissue acquisition for this purpose is modest. Therefore, serum biomarkers are of critical importance to overcome current limitations in the clinical practice. However, CA 19–9 has a high false negative rate and lacks sensitivity and specificity in differentiating PDAC from CP; 40% of patients with CP can exhibit elevated CA 19–9 serum levels [2,18]. Herein, we validate the value of GPC1+crExos coupled to beads to distinguish PDAC from CP with sensitivity and specificity superior to that of CA 19–9. The potential of crExos as biomarkers in PDAC was previously demonstrated [3–10]. Most of these studies compare PDAC with healthy individuals. Our study validates the utility of this biomarker in a pressing clinical problem and can distinguish PDAC from CP patients. Among the initially recruited CP patients, we found that GPC1+crExos were significantly elevated in one MF case. The EUS-guided biopsies revealed malignant transformation, and this patient was included in the PDAC group. Interestingly, a non-MF CP case with high GPC1+crExos presented a *de novo* lesion consistent with a SB-IPMN, while no malignant transformation was observed on follow-up. This could be a false positive result. Nonetheless, there is also the possibility of this information signaling a high-risk individual since GPC1 has been previously shown elevated in some premalignant conditions such as mucinous cystic lesions [5].

Despite the potential of GPC1+crExos as a biomarker, its use in the clinic is still limited by technical constraints. Its translation to the clinic needs a more technically feasible and cost-effective approach to be performed as routine analysis. Unfortunately, the results obtained using ELISA for GPC1+crExos or serum GPC1 do not reflect the data gathered from flow cytometry [4,5]. One of the reasons could be the insufficient amounts of exosomes analyzed. Due to sample size limitations, one-third of the exosomes used for flow cytometry analysis were used for ELISA. Nonetheless, other factors might be at play. Lipid vesicles are present in propofol (2,6-diisopropylphenol) [19] (anesthetic used in some patients during

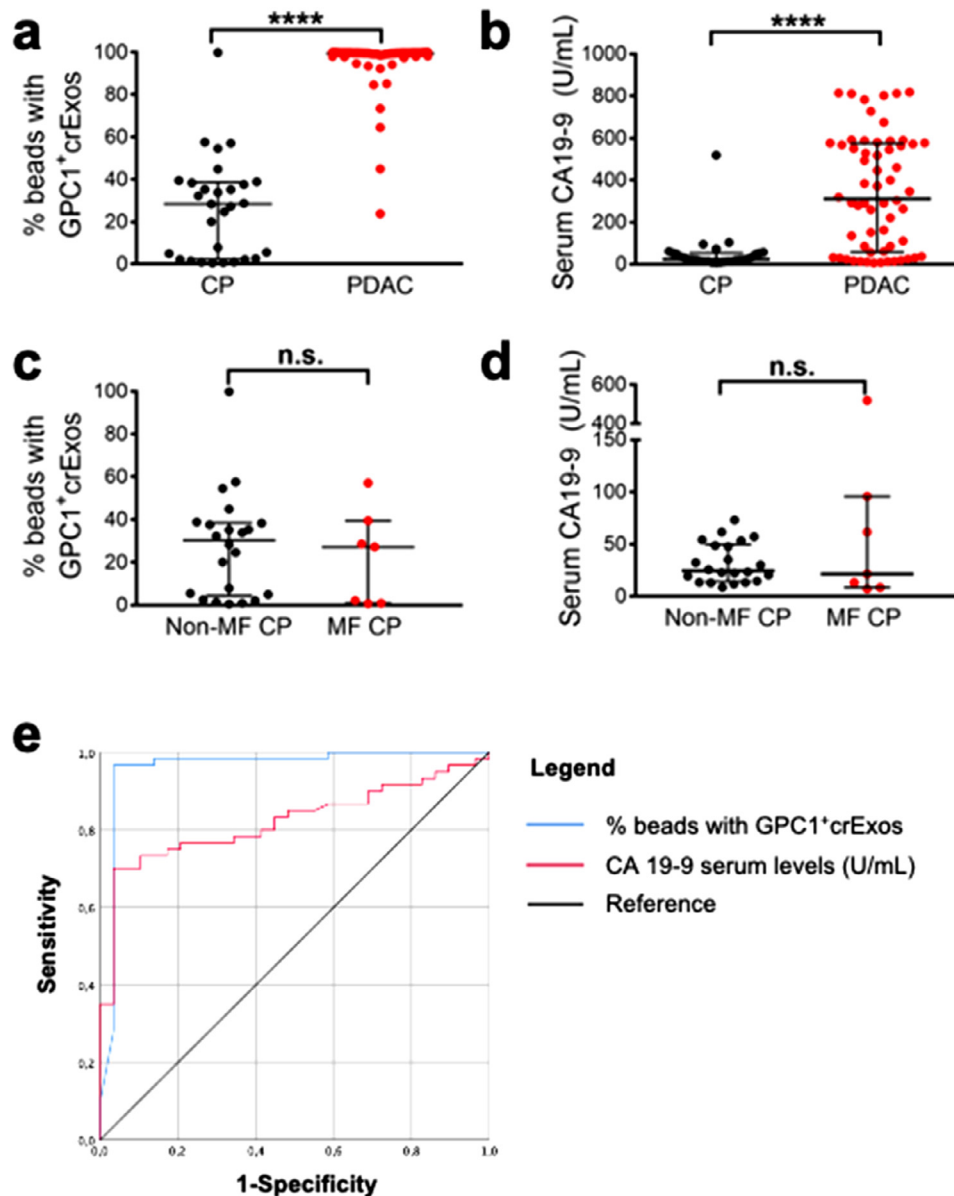


Fig. 2. GPC1+crExos coupled to beads can distinguish PDAC from CP subjects. **a.** Scatter dot plot representing the percentage of beads bound to GPC1-positive exosomes (GPC1+crExos) in CP ($n = 29$) and PDAC ($n = 60$), (Mann-Whitney U Test, **** $p < 0.0001$). **b.** Scatter dot plot representing the serum CA 19-9 concentration (U/mL) as determined by ELISA in CP ($n = 29$) and PDAC ($n = 60$) donors (Mann-Whitney U Test, **** $p < 0.0001$). **c.** Scatter dot plot representing the percentage of beads bound to GPC1+crExos in non-MF CP ($n = 22$) and MF CP ($n = 7$) (Mann-Whitney U Test, n.s. non-significant, $p = 0.533$). **d.** Scatter dot plot representing the serum CA 19-9 concentration (U/mL) determined by ELISA in non-MF CP ($n = 22$) and MF CP ($n = 7$), (Mann-Whitney U Test, n.s. non-significant, $p = 0.901$). **e.** ROC curves of the CP ($n = 29$) and PDAC ($n = 60$) cohort regarding the GPC1+crExos levels (% beads with GPC1+crExos) and CA 19-9 serum levels (U/mL). Data is shown as median $\pm$ interquartile range.

the EUS examination) and could be confused for exosomes. One cannot exclude the possibility that these lipid vesicles introduced bias in the measurement of crExos. Furthermore, the analysis of GPC1+crExos by flow cytometry relies on the use of 4 μ m beads. We cannot control how many exosomes cover one bead, the minimal number of crExos per bead to have a signal, or even the number of GPC1 molecules on each exosome that translates into a positive bead/result.

Despite these facts, new studies have demonstrated the potential of this biomarker using distinct methods of analysis at the protein and mRNA level based on microfluidics [9,10]. Therefore, the development of nanomedicine and nanodevices will greatly support the potential use of crExos as biomarkers in the clinic.

In summary, we show that circulating exosomes positive for GPC1 outperform CA 19-9 as a biomarker to differentiate PDAC from CP. Importantly, we demonstrate that the performance of this biomarker was equally good for both early and advanced stages of the tumor. In addition, we also need to point out some of the limitations associated with our study, namely the lack of power calculation and the small sample size. Therefore, validation of our promising results in future cohorts is necessary. Despite current technical limitations, the potential clinical utility opens new opportunities in the differential diagnosis of PDAC in the context of CP. Additionally, the utility of GPC1+crExos in monitoring response to treatment is still to be determined.

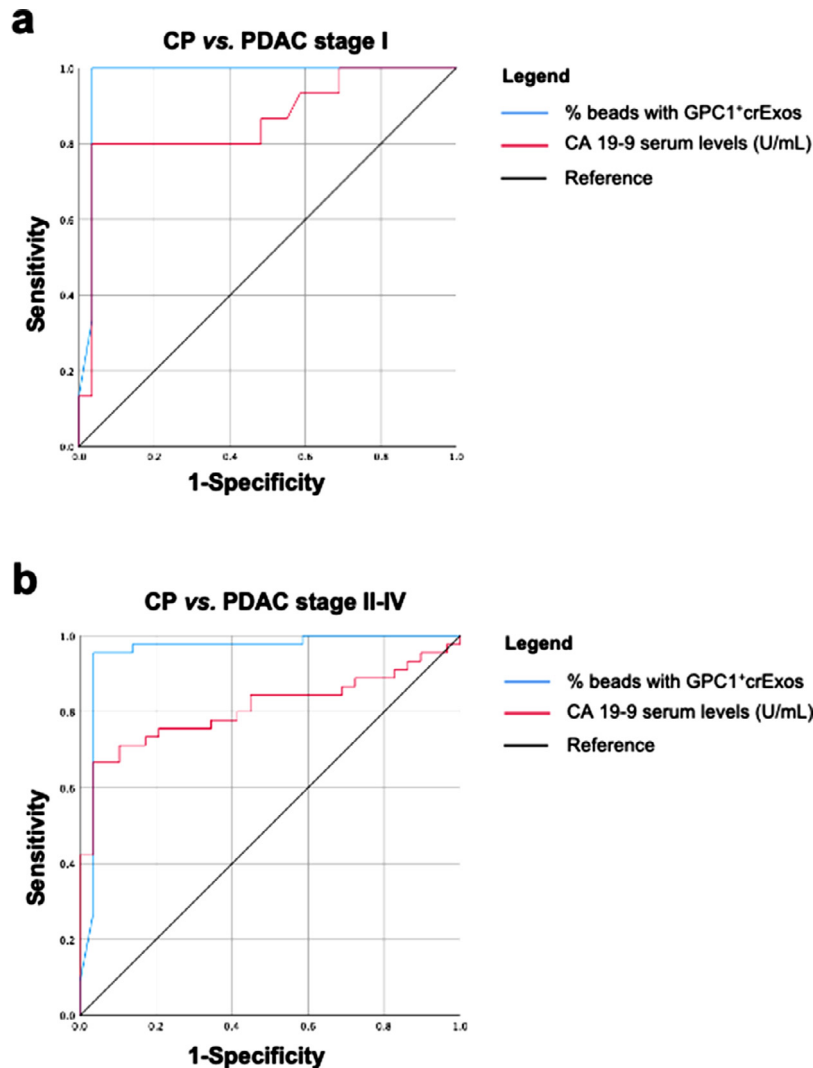


Fig. 3. GPC1+crExos coupled to beads can distinguish patients with CP from early and advanced PDAC stages.

a. ROC curves of the CP ($n = 29$) and PDAC stage I ($n = 15$) cohort regarding the GPC1+crExos levels (% beads with GPC1+crExos) and CA 19-9 serum levels (U/mL). Data is shown as median $\pm$ interquartile range. **b.** ROC curves of the CP ($n = 29$) and PDAC stages II-IV ($n = 45$) cohort regarding the GPC1+crExos levels (% beads with GPC1+crExos) and CA 19-9 serum levels (U/mL). Data is shown as median $\pm$ interquartile range.

Author contribution

Moutinho-Ribeiro P, MD, MSc – main project investigator; patient selection and sample collection; performed pancreatic endoscopic ultrasound, wrote initial manuscript.

Adem B, MSc – samples processing, data analysis, assembling figures and supported manuscript writing.

Batista I, MSc; Silva S, MSc – nanoparticle tracking analysis, flow cytometry samples and ELISA processing.

Silva M, MD – data collection and statistical analysis.

Ruivo CF, MSc – samples processing.

Morais R, MD; Peixoto A, MD; Coelho R, MD; Costa-Moreira, MD – patient observation, data collection and database management.

Lopes S, MD, PhD; Vilas-Boas F, MD – performed pancreatic endoscopic ultrasound.

Durães C, PhD – data analysis.

Lopes J, MD; Barroca H, MD; Carneiro F, MD, PhD – cytological and histological examination of pancreatic specimens.

Melo SA, PhD – supported the conceptual design of the work and supervised data analysis; critical review of the manuscript.

Macedo G, MD, PhD – conceptually designed the work and interpreted data analysis; critical review of the manuscript.

Declaration of Competing Interest

S. A. M. has ownership interests (patents). All other authors disclosed no potential conflicts of interest or financial relationships relevant to this publication.

Informed consent

Informed consent was obtained from all patients included in this study. The study was approved by the ethical committee of Centro Hospitalar Universitário de São João (CHUSJ), Porto, Portugal, under number CES 327-15.

Individual participant data will not be shared.

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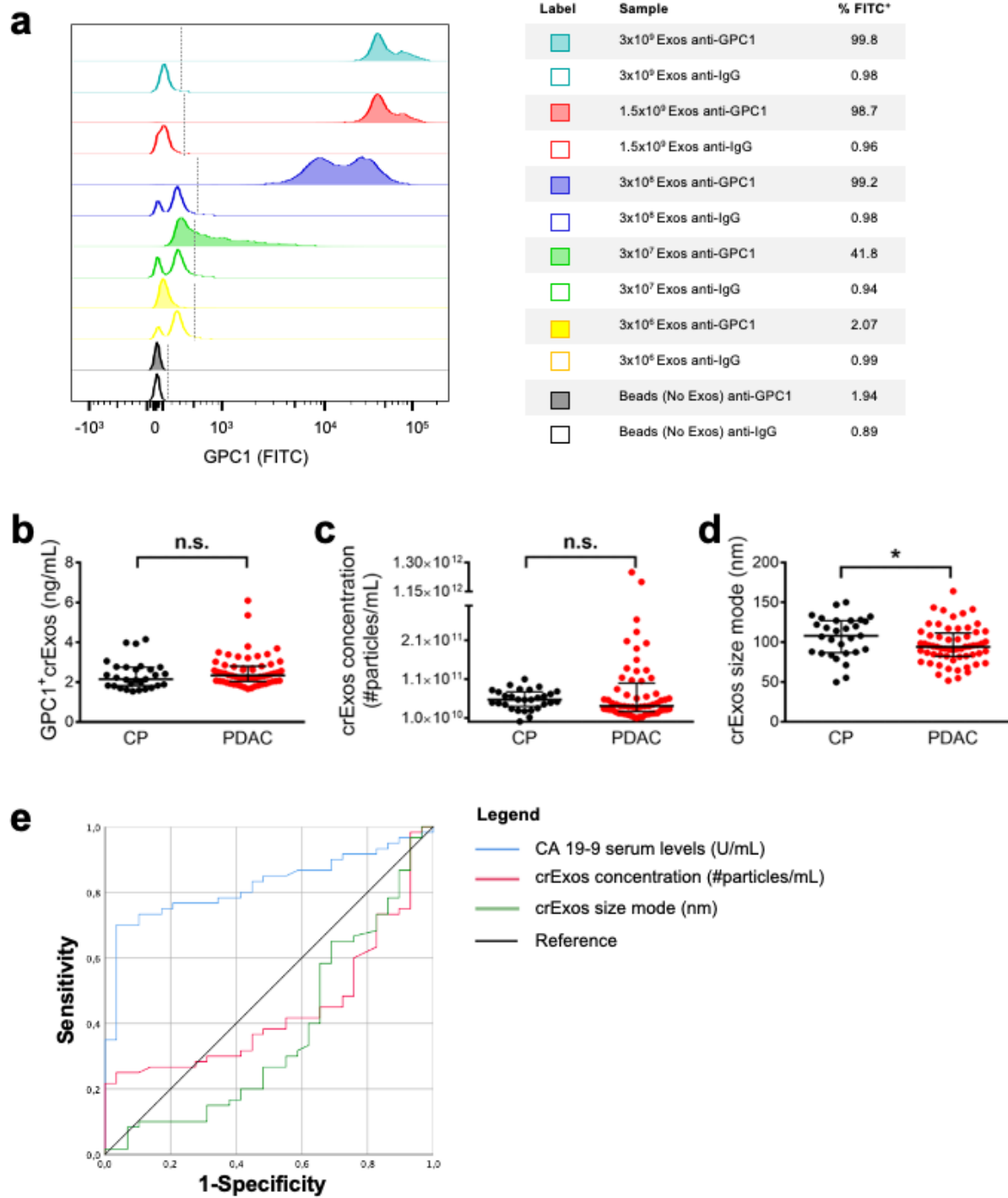
Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.dld.2021.10.012](https://doi.org/10.1016/j.dld.2021.10.012).

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Supplementary Figure 1



Supplementary Figure 1. Size and concentration of crExos do not distinguish PDAC from CP subjects.

a. Histograms depicting a PDAC sample titration experiment. Dashed lines in each condition illustrate the start of the positive gate (anti-IgG (Alexa Fluor 488) > 1% of FITC positivity). Exos=exosomes. **b.** Scatter dot plot representing the serum GPC1⁺crExos concentration (ng/mL) determined by ELISA in CP (n=29) and PDAC (n=60) donors (Mann-Whitney U Test, n.s. non-significant, p=0.161) **c.** Scatter dot plot representing exosomes concentration measured by nanoparticle tracking analysis depicting the number of exosomes per 1 mL of serum derived from CP (n=29) and PDAC (n=60) donors (Mann-Whitney U Test, n.s. non-significant, p=0.356). **d.** Scatter dot plot representing exosomes mode size distribution determined by nanoparticle tracking analysis from 1 mL of serum derived from CP (n=29) and PDAC (n=60) donors (Mann-Whitney U Test, * p=0.047). **e.** ROC curves of the CP (n=29) and PDAC (n=60) cohort regarding the CA 19-9 serum levels (U/mL), crExos concentration (# particles/mL) and crExos size mode (nm). Data is shown as median ± interquartile range.

3.4.4.

PAPER 8

Moutinho-Ribeiro P, Batista I, Quintas S, Adem B, Silva M, Morais R, Peixoto A, Coelho R, Costa-Moreira P, Medas R, Lopes S, Vilas-Boas F, Baptista M, Dias-da-Silva D, Esteves AL, Martins F, Lopes J, Barroca H, Carneiro F, Macedo G, Melo SA.

Exosomal glypican-1 is elevated in pancreatic cancer precursors and can signal genetic predisposition in the absence of endoscopic ultrasound abnormalities.

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Basic Study

Exosomal glypican-1 is elevated in pancreatic cancer precursors and can signal genetic predisposition in the absence of endoscopic ultrasound abnormalities

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Abstract

BACKGROUND

Individuals within specific risk groups for pancreatic ductal adenocarcinoma

(PDAC) [mucinous cystic lesions (MCLs), hereditary risk (HR), and new-late onset diabetes mellitus (NLOD)] represent an opportunity for early cancer detection. Endoscopic ultrasound (EUS) is a premium image modality for PDAC screening and precursor lesion characterization. While no specific biomarker is currently clinically available for this purpose, glypican-1 (GPC1) is overexpressed in the circulating exosomes (crExos) of patients with PDAC compared with healthy subjects or those harboring benign pancreatic diseases.

AIM

To evaluate the capacity of GPC1⁺ crExos to identify individuals at higher risk within these specific groups, all characterized by EUS.

METHODS

This cross-sectional study with a prospective unicentric cohort included 88 subjects: 40 patients with MCL, 20 individuals with HR, and 20 patients with NLOD. A control group (CG) was submitted to EUS for other reasons than pancreatic pathology, with normal pancreas and absence of hereditary risk factors ($n = 8$). The inclusion period was between October 2016 and January 2019, and the study was approved by the Ethics Committee of Centro Hospitalar Universitário de São João, Porto, Portugal. All patients provided written informed consent. EUS and blood tests for quantification of GPC1⁺ crExos by flow cytometry and carbohydrate antigen 19-9 (CA 19-9) levels by ELISA were performed in all subjects. EUS-guided tissue acquisition was done whenever necessary. For statistical analysis, SPSS® 27.0 (IBM Corp., Armonk, NY, United States) version was used. All graphs were created using GraphPad Prism 7.00 (GraphPad Software, San Diego, CA, United States).

RESULTS

Half of MCLs harbored worrisome features (WF) or high-risk stigmata (HRS). Pancreatic abnormalities were detected by EUS in 10.0% and 35.0% in HR and NLOD individuals, respectively, all considered non-malignant and “harmless.” Median levels of GPC1⁺ crExos were statistically different: MCL [99.4%, interquartile range (IQR): 94.9%-99.8%], HR (82.0%, IQR: 28.9%-98.2%), NLOD (12.6%, IQR: 5.2%-63.4%), and CG (16.2%, IQR: 6.6%-20.1%) ($P < 0.0001$). Median levels of CA 19-9 were within the normal range in all groups (standard clinical cut-off of 37 U/mL). Within HR, individuals with a positive history of cancer had higher median levels of GPC1⁺ crExos (97.9%; IQR: 61.7%-99.5%), compared to those without (59.7%; IQR: 26.3%-96.4%), despite no statistical significance ($P = 0.21$). Pancreatic cysts with WF/HRS were statistically associated with higher median levels of GPC1⁺ crExos (99.6%; IQR: 97.6%-99.8%) compared to those without (96.5%; IQR: 81.3%-99.5%) ($P = 0.011$), presenting an area under the receiver operating characteristic curve value of 0.723 (sensitivity 75.0% and specificity 67.7%, using a cut-off of 98.5%; $P = 0.012$).

CONCLUSION

GPC1⁺ crExos may act as biomarker to support the diagnosis and stratification of PDAC precursor lesions, and in signaling individuals with genetic predisposition in the absence of EUS abnormalities.

Key Words: Glypican-1; Circulating exosomes; Endoscopic ultrasound; Pancreatic cancer risk groups; Pancreatic cancer precursor lesions; Genetic predisposition

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Core Tip: Patients with mucinous cystic lesions (MCLs), hereditary risk (HR), and new-late onset diabetes mellitus represent a target for early pancreatic ductal adenocarcinoma (PDAC) detection. Within these groups, we evaluated the capacity of glypican-1 positive (GPC1⁺) circulating exosomes (crExos) to identify individuals at higher risk, all characterized by endoscopic ultrasound (EUS). High levels of GPC1⁺ crExos were present in MCL and in individuals with HR (predominantly in those with history of cancer), even in the absence of EUS abnormalities. GPC1 may represent a biomarker to support the diagnosis and stratification of PDAC precursor lesions, and indicate genetic predisposition.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is considered one of the deadliest malignant diseases around the world, and is estimated to become the second leading cause of cancer-related deaths in the United States in 2030[1]. Most patients present with advanced disease at diagnosis, with only 20% being candidates for surgical treatment, the only chance for cure[2,3].

In its early stages, PDAC usually develops with few or no symptoms, and current sectional imaging modalities are inadequate to detect early small lesions[3]. Endoscopic ultrasound (EUS) is a highly accurate diagnostic technique for pancreatic lesions, with its role majored by the possibility of performing EUS-guided tissue acquisition[4-6].

Among PDAC precursor lesions are pancreatic intraepithelial neoplasias (PanINs) and mucinous cystic lesions (MCLs)[7,8]. While the former are very difficult to identify by available imaging modalities, the latter can be more clearly detected and characterized, specially by magnetic resonance imaging (MRI)/magnetic resonance cholangiopancreatography (MRCP) and EUS[5,9].

Along with the improvement of imaging diagnostic accuracy, the search for a biomarker that could adequately identify PDAC at early stages or its high-risk precursor lesions is a top research priority. The only biomarker approved for clinical use is carbohydrate antigen 19-9 (CA 19-9), but it lacks sensitivity and specificity for PDAC early detection, being mostly reserved for monitoring response to therapy and disease progression[10]. In recent years, several molecules have been tested to serve this purpose[11,12]. Among these, membrane-anchored proteoglycan glypican-1 (GPC1) has been shown to be a good candidate. We previously studied GPC1 in circulating exosomes (crExos) and found that it could identify PDAC patients among healthy individuals with perfect accuracy[13]. In addition, GPC1⁺ crExos correlated with tumor burden and patients' survival. Moreover, in a genetically engineered mouse model of PDAC, GPC1 was overexpressed in crExos even before the tumor could be detected by MRI [13]. Finally, we also showed higher levels of GPC1⁺ crExos in MCL compared to controls, although the number of patients was very limited[13]. Several studies have demonstrated the role of GPC1 in PDAC [14-18]. Considering the distinct methodologies that some of the studies adopted to study GPC1 in circulation, some support the potential of GPC1⁺ crExos as a biomarker for early detection of PDAC[14, 15,19], while others only demonstrate their correlation with disease burden[16-18].

At present, while 90% of PDAC cases are sporadic, only individuals harboring increased hereditary risk (HR), either kindreds of familial pancreatic cancer (FPC) (7%) or belonging to other cancer syndromes with increased risk of PDAC (3%), are candidates for screening[20-22]. Nevertheless, there are other well-defined PDAC-associated risk groups that deserve special attention and can constitute a refined population to be surveilled in order to increase the rate of early detection and improve overall survival. One of these groups is composed by individuals older than 50 years with a recent (< 36 mo) diagnosis of type II diabetes mellitus (DM). Increasing epidemiological, clinical, and experimental evidence shows that new-onset DM can be a clinical manifestation of asymptomatic PDAC or harbinger the disease and offers the promise for early detection in these individuals[3,23-26]. In fact, although the complex and multidirectional relationship between the two entities is not fully understood, new-late onset DM (NLOD) has been recognized as an entity signaling a 6- to 8-fold increased risk of developing PDAC in 3 years[27].

We previously showed that levels of GPC1⁺ crExos discriminate PDAC from chronic pancreatitis (CP) with high accuracy[28]. In this work, we aimed to determine the capacity of GPC1⁺ crExos to identify individuals at higher risk of developing PDAC, comparing its levels with EUS pancreatic abnormalities (PA) within specific risk groups: MCL, HR, and NLOD.

MATERIALS AND METHODS

Design of the study and population

This cross-sectional study with a prospective unicentric cohort included 88 subjects: 40 patients with MCLs, 20 individuals with HR, 20 patients with NLOD, and 8 individuals in the control group (CG). The inclusion period was between October 2016 and January 2019, and the study was approved by the Ethics Committee of Centro Hospitalar Universitário de São João (CHUSJ) (ID No. CES 327-15), Porto,

Portugal. All patients provided written informed consent and underwent blood sample collection at the time of EUS.

In the MCL group, we considered for inclusion both intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). For its diagnosis, we used both imagiological and cyst fluid analysis criteria. We have assumed IPMN etiology when EUS and/or MRCP clearly showed communication of the cyst(s) with the pancreatic ductal system. In this situation, branch-duct type (BD-IPMN) were presumed if multiple and no dominant main pancreatic duct (MPD) dilation was seen. Main-duct IPMN (MD-IPMN) was considered if a segmental or diffuse dilatation of the main duct > 5 mm was observed without any other cause of obstruction. Mixed-type IPMN (MT-IPMN) was defined when features of MD-IPMN and BD-IPMN coexisted[29]. MCN was assumed when a mucin-producing cyst forming epithelial neoplasia of the pancreas with a distinctive ovarian-type stroma was present, typically with no communication with the ductal system[30].

We performed EUS-guided fine needle aspiration (FNA) in almost all cystic lesions included. Mucinous nature was supported by a fluid carcinoembryonic antigen (CEA) > 192 ng/mL and/or fluid glucose < 50 mg/dL[31]. In relation to amylase content, if > 250 U/L, a diagnosis of IPMN was likely, whereas levels < 250 U/L suggested MCN. If the aspirated content was sufficient, a sample was also evaluated by experienced cytopathologists.

In the HR group, we considered for inclusion FPC (family history of PDAC in at least two first-degree, or in three or more first- and second-degree relatives) and PDAC susceptibility gene mutation carriers[20,22,32,33]. All of these individuals had a clinically and genetically established diagnosis and have been followed in a dedicated consultation for hereditary digestive cancers in our institution. They underwent detailed evaluation of family history, and verification of cancer diagnoses by review of medical records and genetic testing.

In the NLOD group, we included patients aged ≥ 50 years who had been diagnosed with type II DM within a period of 36 mo[23,24]. Diagnosis of type II DM was made according to the American Diabetes Association and consisted of: Fasting plasma glucose level ≥ 126 mg/dL, a 2-h plasma glucose level of ≥ 200 mg/dL during a 75-g oral glucose tolerance test, a random plasma glucose level of ≥ 200 mg/dL in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, or a hemoglobin A1c level of $\geq 6.5\%$ [34].

In the CG, individuals who underwent EUS for other reasons than pancreatic pathology were included with a normal pancreas and absence of hereditary/familial risk factors. The exclusion criteria were patients unable or unwilling to give informed consent, individuals younger than 18 years of age, pregnancy or breast feeding, contraindications to endoscopic procedures or contrast administration, contraindications to computed tomography (CT) or MRI, coagulopathy (prothrombin time $> 50\%$ of control, activated partial thromboplastin time > 50 s, or international normalized ratio > 1.5), patients on chronic anticoagulation, platelet count $< 50000/\mu\text{L}$, and inability to tolerate sedated upper endoscopy due to cardiopulmonary instability or other contraindication to endoscopic procedures.

At the time of inclusion, immediately before EUS examination, a blood sample was collected for complete blood count as well as renal, liver, and pancreatic chemistry at the CHUSJ laboratory. A separate blood sample collected at the same time was used to quantify CA 19-9 serum levels (ab108642, CA 19-9 Human ELISA Kit; Abcam, Cambridge, MA, United States) and GPC1 expression in crExos by fluorescence-activated cell sorting (FACS) (Figure 1). For CA 19-9, the standard clinical cut-off of 37 U/mL was used. A complete personal and family clinical history was registered, and demographic data was recorded [age, body mass index (BMI)], previous history of pancreatic disease, smoking and drinking habits, history of diabetes and duration since diagnosis, digestive and systemic symptoms. Laboratory values and previous results of cross-sectional imaging (CT, MRI, or magnetic resonance cholangiopancreatography) were registered. The results of the EUS evaluation at the time of inclusion in the study as well as transechoendoscopic or surgically collected specimens were also recorded.

All subjects were observed, examined, and followed-up at the Department of Gastroenterology of CHUSJ (Porto, Portugal) until death or July 2021.

EUS, tissue acquisition techniques, and fluid/specimen analyses

All EUS evaluations were performed by three experienced endoscopists under deep sedation (propofol or pethidine plus midazolam as assisted by an anesthesiologist). Linear scopes from Olympus® (GT-UCT140, GT-UCT180; Tokyo, Japan) and Pentax® (EG-3670UTK, EG-3870UTK; Tokyo, Japan) along with Olympus® EU-ME2 and Hitachi Avius® (Tokyo, Japan) image processors were used.

PA were classified into major and minor changes. Major changes comprised cystic lesions, CP-like parenchymal changes, and solid lesions. Minor ones constituted changes in pancreatic echogenicity compatible with lipomatous transformation[35,36]. EUS high-risk stigmata (HRS) and worrisome features (WF) were categorized according to the International consensus of Fukuoka guidelines for the management of IPMN of the pancreas[29]. A main duct dilatation ≥ 10 mm or an enhancing mural nodule > 5 mm were classified as HRS, whereas a main duct dilatation between 5 and 9 mm, an enhancing mural nodule < 5 mm, a cyst diameter > 3 cm, the presence of thickened/enhancing cyst walls, an abrupt change in pancreatic duct caliber with distal pancreatic atrophy, and lymphadenopathy were considered WF.

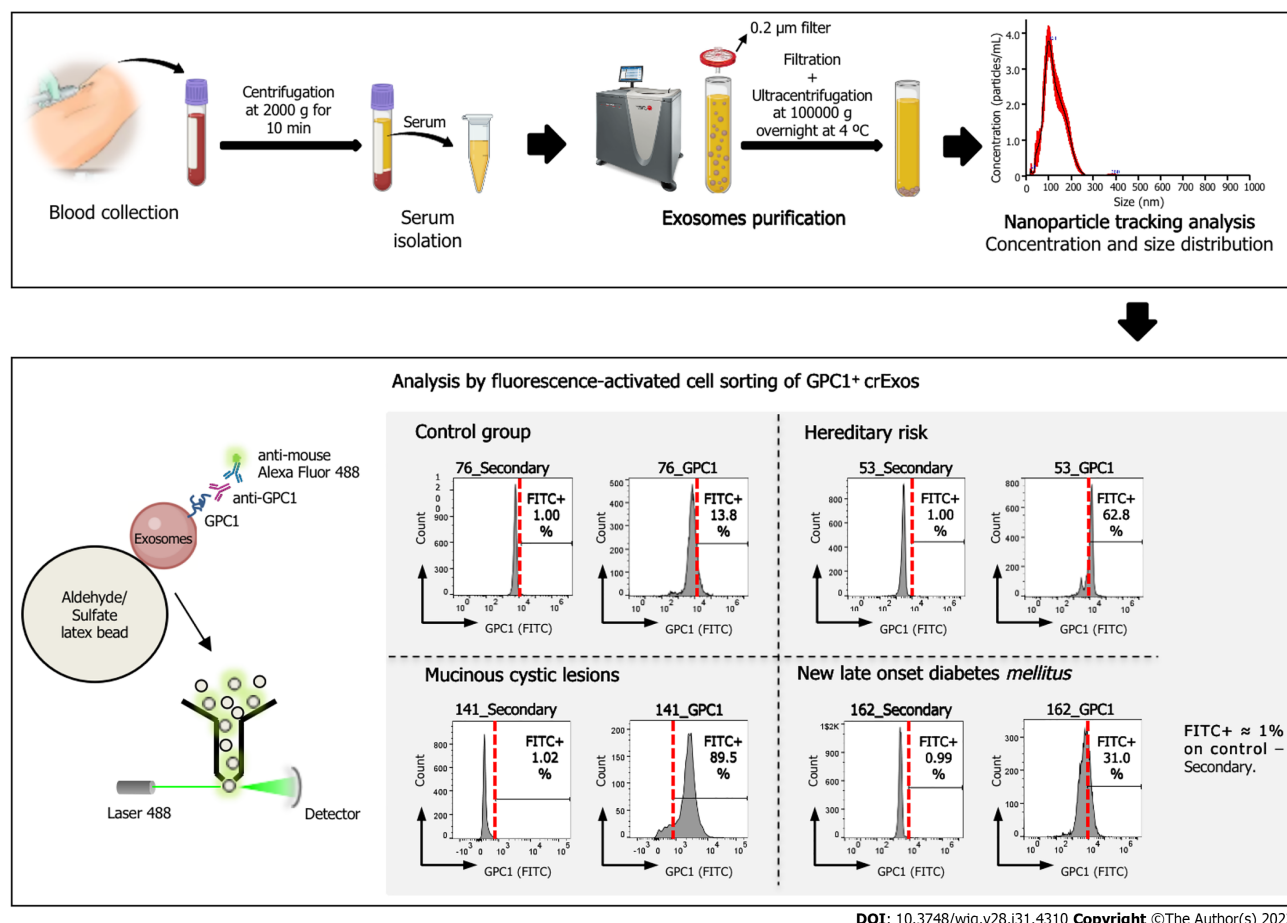


Figure 1 Schematic representation of the workflow for the analysis of circulating exosomes from control group, mucinous cystic lesions, hereditary risk and new-late onset diabetes mellitus patients for the expression of glypican-1. Scheme illustrating the workflow of our study of the expression of glypican-1 (GPC1) on exosomes retrieved from patients' blood samples, from blood collection to the analysis by flow cytometry of GPC1+ circulating exosomes (crExos). A representative histogram of the percentage of beads bound to GPC1-positive crExos (GPC1+ crExos) that were isolated from the serum of a control group, composed by individuals submitted to endoscopic ultrasound for other reasons than pancreatic pathology, as well as the following pancreatic ductal adenocarcinoma risk groups: Mucinous cystic lesions, hereditary risk, and new-late onset diabetes mellitus. Red dashed lines in each histogram depict the start of the fluorescein isothiocyanate (FITC)-positive gate (anti-immunoglobulin G) Alexa Fluor 488), which was determined for each patient separately by considering FITC+ approximately 1% on control–secondary–but maintained between control and GPC1 samples. GPC1: Glypican-1; FITC: Fluorescein isothiocyanate; crExos: Circulating exosomes.

Doppler, elastography, and contrast-enhanced EUS image acquisition (sulphur hexafluoride microbubbles; SonoVue®, Bracco, Italy) were used for assistance in the characterization of mural nodules and thickened cyst walls.

EUS-FNA was performed in almost all cystic lesions, preferably with a 22 G needle (Wilson-Cook Medical, Winston, NC, United States and Boston Scientific, Natick MA, United States) in a single pass, and along with antibiotic prophylaxis (single intravenous infusion of 200 mg ciprofloxacin). The fluid collected was sent for amylase, glucose, and CEA quantification, and also, if in enough volume, for cytological examination. Solid or indeterminate lesions, mural nodules, or suspicious thickenings of the cyst wall or septa were punctured in a distinct pass, preferably with a 22 G fine needle biopsy. Adverse events related to EUS procedures were registered and monitored.

Microscopic examinations of specimens resulting from EUS-FNA/B and/or surgical resection were analyzed by experienced pathologists in pancreatic diseases and contextualized to patients' clinical history and imaging findings. Cytological evaluation was performed according to the Papanicolaou Society of Cytopathology System for Reporting Pancreaticobiliary Cytology[37].

Collection of human blood samples

At the same time of EUS performance, blood samples were collected at the Department of Gastroenterology of CHUSJ. Serum samples were obtained by centrifugation of the whole blood sample at 2000 g for 10 min at 4 °C and collection of the supernatant. The resultant serum samples were then aliquoted and stored at -80 °C prior to analysis.

Isolation of exosomes by ultracentrifugation of serum samples and nanoparticle tracking analysis

Human serum samples were allowed to thaw on ice with periodic agitation to avoid degradation. A volume of 200 μ L serum was retrieved into a new tube and centrifuged at 10000 rpm for 2 min at 4 °C. The recovered supernatant was diluted in 200 μ L NaCl prior to filtration into 14 mL Open-Top Thinwall Ultra-Clear Tubes (Beckman Coulter, Inc., Brea, CA, United States) using a 0.2 μ m pore filter (Whatman International Ltd., England, United Kingdom). The tubes were filled with NaCl, and the samples were ultracentrifuged at 100000 g overnight at 4 °C. The next day, the supernatant was carefully and thoroughly discarded and the exosomes' pellet was resuspended in 300 μ L of 1 $\times$ phosphate-buffered saline (PBS). To determine particle concentration and size distribution, nanoparticle tracking (NTA) (NanoSight NS300) was performed using 10 μ L of the exosomes sample dissolved in 1 $\times$ PBS at a 1:100 dilution. The remaining exosomes sample was saved at -20 °C for downstream analysis.

GPC1 analysis by FACS of exosomes coupled to beads

Using the exosomes concentration previously obtained by NTA analysis as a reference, an equal number of exosomes was used for downstream FACS analysis. A total of 3.0×10^9 exosomes were coupled to 4 μ m aldehyde/sulfate latex beads (A37304; Thermo Fisher Scientific, Waltham, MA, United States) that had been previously equilibrated at room temperature (through resuspension of 5 μ L beads in 100 μ L of 1 $\times$ PBS and rotation at room temperature for 15 min). The sample volume was adjusted to 300 μ L with 1 $\times$ PBS. Upon incubation in a rotator at room temperature for 15 min and then at 4 °C for 30 min to allow the formation of exosomes-bead complexes, 300 μ L glycine 1 M in 1 $\times$ PBS was added to the sample, followed by incubation at room temperature for 1 h with continuous rotation. Samples were centrifuged at 12000 rpm for 2 min and the supernatant was discarded. The pellet containing the exosomes-bead complexes was subjected to a blocking step with 100 μ L of 10% bovine serum albumin (BSA) in 1 $\times$ PBS and then incubated with continuous rotation at room temperature for 45 min. The samples were centrifuged at 12000 rpm for 2 min and divided into two tubes: Control and GPC1-incubated samples. Control samples were incubated with a solution of 2% BSA in 1 $\times$ PBS, whereas experimental samples were incubated with anti-GPC1 (1:240 dilution in a solution of 2% BSA in 1 $\times$ PBS, MAB8351; Abnova, Taipei, Taiwan) overnight in a rotator at 4 °C. The next morning, the samples were spun at 12000 rpm for 2 min and the pellet was washed twice with 2% BSA in 1 $\times$ PBS, with centrifugation at 12000 rpm for 2 min between washes. Following incubation with an Alexa-488-tagged secondary antibody (anti-mouse A-21202; Thermo Fisher Scientific) for 30 min with continuous rotation at room temperature of both control and experimental samples, samples were washed again twice with 2% BSA in 1 $\times$ PBS. Finally, samples were resuspended in 300 μ L of 2% BSA in 1 $\times$ PBS for BD Accuri C6 or BD FACS Canto II analysis (BD Biosciences, Haryana, IN, United States). Using the control samples of each patient (*i.e.* exosomes-bead complexes from each patient only incubated with secondary antibody) as a reference, the fluorescein isothiocyanate (FITC) voltage was adjusted until the percentage of FITC⁺ beads was 1%. Then, using the same gate and FITC voltage, the percentage of beads bound with GPC1⁺ crExos in the experimental sample was determined for each patient separately. Data were analyzed using FlowJo software.

Statistical analyses

Categorical variables are described as absolute and relative frequencies and continuous variables as mean and standard deviation, median, percentiles, minimum, and maximum. Hypotheses were tested regarding the distribution of continuous variables *via* the independent samples *t*-test/one-way analysis of variance (ANOVA) or nonparametric Mann-Whitney and Kruskal-Wallis test depending on normal or non-normal distribution, respectively, and considering the nature of the hypothesis. The chi-squared and ANOVA tests were used for categorical variables analysis. Pearson's correlation coefficient was used to assess the statistical relationship/association between two continuous variables. The diagnostic accuracy of GPC1⁺ crExos was assessed by receiver operating characteristic curve (ROC) considering 95% confidence intervals. Area under the ROC curve (AUROC) was calculated. SPSS® 27.0 (IBM Corp., Armonk, NY, United States) version was used. All graphs were created using GraphPad Prism 7.00 (GraphPad Software, San Diego, CA, United States). The statistical review of the study was performed by a biomedical statistician.

RESULTS

Population demographic characteristics

The baseline demographic characteristics of the 88 individuals included in the study (40 MCL, 20 HR, 20 NLOD, and 8 CG) are summarized in Table 1. The overall female:male ratio was 1.6:1 and did not differ statistically among groups. The mean age of the population was 60.3 ± 12.6 years. The older individuals belonged to the MCL group, with a median age of 67.2 ± 8.9 years, whereas the younger individuals were in the HR group, with a median age of 48.7 ± 10.8 years ($P < 0.001$). Patients in the NLOD group presented with a significantly higher BMI (29.9 ± 4.5 kg/m²; $P < 0.001$).

Table 1 Baseline demographic characteristics of the study population, *n* = 88

Cohort characteristics	MCL, <i>n</i> = 40	HR, <i>n</i> = 20	NLOD, <i>n</i> = 20	CG, <i>n</i> = 8	Total, <i>n</i> = 88	<i>P</i> value
Sex						NS
Male	14 (35.0)	9 (45.0)	9 (45.0)	2 (25.0)	34 (38.6)	
Female	26 (65.0)	11 (55.0)	11 (55.0)	6 (75.0)	54 (61.4)	
Age in yr	67.2 ± 8.9	48.7 ± 10.8	60.8 ± 7.4	53.8 ± 18.7	60.3 ± 12.6	< 0.001
BMI in kg/m ²	24.9 ± 3.8	24.2 ± 3.9	29.9 ± 4.5	25.0 ± 4.0	25.9 ± 4.5	< 0.001
Smoking habits						NS
Never	27 (67.5)	13 (65.0)	12 (60.0)	7 (87.5)	59 (67.1)	
Ex-smoker, > 5 yr	8 (20.0)	4 (20.0)	7 (35.0)	1 (12.5)	20 (22.7)	
Active	5 (12.5)	3 (15.0)	1 (5.0)	0 (0.0)	9 (10.2)	
Alcohol habits						NS
No	22 (55.0)	11 (55.0)	10 (50.0)	7 (87.5)	50 (56.8)	
Yes	18 (45.0)	9 (45.0)	10 (50.0)	1 (12.5)	38 (43.2)	
Family history of PDAC						< 0.001
No	34 (85.0)	7 (35.0)	18 (90.0)	8 (100.0)	67 (76.1)	
Yes	6 (15.0)	13 (65.0)	2 (10.0)	0 (0.0)	21 (23.9)	

Data are presented as *n* (%) or mean ± SD. BMI: Body mass index; CG: Control group; HR: Hereditary risk; MCL: Mucinous cystic lesion; NLOD: New-late onset diabetes mellitus; NS: Non-significant; PDAC: Pancreatic ductal adenocarcinoma; SD: Standard deviation.

No statistically significant differences were observed in relation to smoking or drinking habits among the studied groups. Overall, 10.2% of the population were active smokers and 22.7% ex-smokers (more than 5 years of abstinence). In relation to alcohol consumption, 43.2% were considered active drinkers (average ingesting amounts of > 30 g or > 40 g alcohol *per* day, in case of female or male individuals, respectively).

Family history of PDAC was present in 23.9% of the entire population, with the highest proportion observed in the HR group (65.0%; *P* < 0.001). In the HR group, 17 (85.0%) individuals were diagnosed with Lynch Syndrome, 1 (5.0%) with Peutz-Jeghers syndrome, and the remaining 2 (10%) with FPC. Six individuals with Lynch Syndrome had a previous personal history of cancer, mostly colorectal (*n* = 5), but all were disease-free at the time of inclusion. Detailed information about this group, including the type of harbored mutation(s), can be found in [Table 2](#). In the NLOD group, the mean time between inclusion in the study and establishment of DM diagnosis was 20.9 ± 8.6 mo.

EUS findings and other clinicopathological features

Main PA detected by EUS is illustrated in [Figure 2A-F](#) and constituted cystic lesions (with or without WF/HRS), CP-like parenchymal changes, and lipomatous transformation of pancreatic parenchyma. The EUS features of cystic lesions in the MCL group are described in [Table 3](#). Of the 40 cases included, 39 (97.5%) were IPMNs, and most (89.8%) were classified as BD-IPMNs. Sixty percent of patients presented with multiple cysts, with a mean size of the dominant lesion of 28.1 ± 14.1 mm. In respect to WF and/or HRS, mural nodules, wall thickening, and a dilated MPD were identified in 10%, 7.5%, and 15% of the lesions, respectively. Nineteen of the forty MCL patients (47.5%) underwent additional study with MRCP.

The clinicopathological characteristics of the study population, particularly the type of PA found in EUS among the different groups, the results of EUS-guided tissue acquisition, surgical treatment and specimen analysis, as well as information on cancer detection during follow-up and mortality rate, are summarized in [Table 4](#). Besides the MCL group, where PA in the form of cyst(s) were present in all patients by definition, PA were also detected during the EUS exam in 10.0% and 35.0% of the subjects belonging to HR and NLOD groups, respectively. Half of the lesions in the MCL group were considered to harbor WF or HRS, in contrast to the PA found in the other groups that were all “harmless” in terms of ultrasonographic appearance (CP-like parenchymal changes, lipomatous transformation and infracentimetric simple cystic lesions). EUS-FNA/B was performed only in the MCL group in 35 of the 40 patients (87.5%), either to confirm the diagnosis or in the presence of WF or HRS ([Figure 2G and H](#)). Malignancy was detected in 3 patients; in 1, the malignancy was detected in a subsequent EUS exam performed during follow-up (this case was previously reported by our team[38]). The result of cytological exam was considered inconclusive in 37.1% of the procedures. Of the 88 patients of the

Table 2 Characterization of hereditary risk group: Germline mutations and personal history of cancer, *n* = 20

	Lynch syndrome, <i>n</i> = 17 (85.0%)	Peutz-Jeghers syndrome, <i>n</i> = 1 (5.0%)	FPC, <i>n</i> = 2 (10.0%)
Germline mutation(s)			
<i>MLH1</i>	7 (41.2)	-	-
<i>MSH2</i>	6 (35.3)	-	-
<i>MLH1</i> + <i>MSH2</i>	1 (5.9)	-	-
<i>MSH2</i> + <i>MSH6</i>	2 (11.7)	-	-
<i>MSH6</i>	1 (5.9)	-	-
<i>STK11</i>	-	1 (100.0)	-
Not identified	-		2 (100.0)
Personal history of cancer			
No	11 (64.7)	1 (100.0)	2 (100.0)
Yes	6 (35.3)	0 (0.0)	0 (0.0)
Type of cancer			
Colon and rectum	5 (83.3%)	-	-
Endometrium	1 (16.7)	-	-

FPC: Familial pancreatic cancer.

Table 3 Endoscopic ultrasound features of cystic lesions among the mucinous cystic lesions group, *n* = 40

Type of mucinous cyst, <i>n</i> (%)	MCN	1 (2.5)
	IPMN	39 (97.5)
Type of IPMN, <i>n</i> (%)	MD-IPMN	2 (5.1)
	BD-IPMN	35 (89.8)
	MT-IPMN	2 (5.1)
Number of cysts, <i>n</i> (%)	Single	16 (40.0)
	Multiple	24 (60.0)
Main cyst size in mm, mean ± SD		28.1 ± 14.4
Main cyst size in mm, median (IQR)		21.5 (16.3-40.5)
Septa, <i>n</i> (%)	Absent	10 (25.0)
	Present	30 (75.0)
Mural nodules, <i>n</i> (%)	Absent	36 (90.0)
	Present	4 (10.0)
Wall thickening, <i>n</i> (%)	Absent	37 (92.5)
	Present	3 (7.5)
MPD caliber, <i>n</i> (%)	Normal	34 (85.0)
	Dilated	6 (15.0)

BD-IPMN: Branch-duct IPMN; IPMN: Intraductal papillary mucinous neoplasm; IQR: Interquartile range; MCN: Mucinous cystic neoplasm; MD-IPMN: Main-duct IPMN; MPD: Main pancreatic duct; MT-IPMN: Mixed type.

cohort, 15 (17.0%) were submitted to surgical resection, and all belonged to the MCL group. These 15 patients either harbored MD or MT-IPMNs or presented a cyst with WF/HRS with suspicious or positive cytology. Malignancy was confirmed in the surgical specimen in all 3 patients with a previous positive cytological exam, and the remaining 12 had a definitive histopathological diagnosis of a low grade dysplastic MCL.

Table 4 Clinical-pathological findings, treatment and follow-up of the study population, *n* = 88

	MCL, <i>n</i> = 40	HR, <i>n</i> = 20	NLOD, <i>n</i> = 20	CG, <i>n</i> = 8	Total, <i>n</i> = 88
PA in EUS					
No	0 (0.0)	18 (90.0)	13 (65.0)	8 (100.0)	39 (44.3)
Yes	40 (100.0)	2 (10.0)	7 (35.0)	0 (0.0)	49 (55.7)
Type of PA					
Cystic lesions	40 (100.0)	2 (100.0)	2 (28.6)	-	44 (89.8)
CP-like parenchymal changes	0 (0.0)	0 (0.0)	2 (28.6)	-	2 (4.1)
Solid lesions	0 (0.0)	0 (0.0)	0 (0.0)	-	0 (0.0)
Lipomatous transformation	0 (0.0)	0 (0.0)	3 (42.8)	-	3 (6.1)
PA with HRS / WF					
No	20 (50.0)	2 (100.0)	7 (100.0)	-	29 (59.2)
Yes	20 (50.0)	0 (0.0)	0 (0.0)	-	20 (40.8)
EUS FNA/B					
No	5 (12.5)	20 (100.0)	20 (100)	8 (100)	53 (60.2)
Yes	35 (87.5)	0 (0.0)	0 (0.0)	0 (0.0)	35 (39.8)
Inconclusive	13 (37.1)	-	-	-	13 (37.1)
Benign	19 (54.3)	-	-	-	19 (54.3)
Malignant	3 (8.6) ¹	-	-	-	3 (8.6) ¹
Surgery					
No	25 (62.5)	20 (100)	20 (100)	8 (100)	73 (83.0)
Yes	15 (37.5)	0 (0.0)	0 (0.0)	0 (0.0)	15 (17.0)
Benign/Malignant	12 (80.0)/3 (20.0)	-/-	-/-	-/-	12 (80.0)/3 (20.0)
F-up in mo	33.3 ± 12.8	34.9 ± 7.1	27.7 ± 4.7	36.1 ± 5.1	32.6 ± 10.0
Cancer detection during F-up	1 (2.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)
Mortality	5 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	5 (5.7)

Data are presented as *n* (%) or mean ± SD.

¹One of 3 patients with malignant cytology was diagnosed during follow-up.

CG: Control group; CP: Chronic pancreatitis; EUS: Endoscopic ultrasound; FNA/B: Fine needle aspiration/biopsy; F-up: Follow up; HR: Hereditary risk; HRS: High risk stigmata; MCL: Mucinous cystic lesion; NLOD: New-late onset diabetes mellitus; NS: Non-significant; PA: Pancreatic abnormality; WF: Worrisome features.

The mean follow-up period of the entire cohort was 32 ± 8.6 mo and did not differ statistically among groups (*P* = NS). Five of the 88 individuals (5.7%) died during follow-up, but only one of these due to cancer progression. Two of the other deaths were related to post-operative complications in a 74-year-old and 75-year-old patients, one was related to a pulmonary carcinoma that developed 1 year after inclusion in a 72-year-old patient, and the remaining one corresponded to an 85-year-old patient that did not survive to an infectious respiratory insufficiency. The 2 other patients submitted to surgical resection of malignant cysts were alive and disease-free after 45 mo of follow-up.

Exosomal GPC1 levels are elevated in MCL lesions with WF/HRS and HR groups

The overall median levels of GPC1⁺ crExos coupled to beads were statistically different among the studied groups: MCL [99.4%, interquartile range (IQR): 94.9%-99.8%], HR (82.0%, IQR: 28.9%-98.2%), and NLOD and CG groups (12.6%, IQR: 5.2%-63.4% and 16.2%, IQR: 6.6%-20.1%, respectively) (*P* < 0.0001) (Figure 3A and Table 5).

The crExos concentration and their median size were significantly higher in NLOD patients (6.05E+10/mL; IQR: 4.83-7.22 and 100.7 nm; IQR: 89.2-110.1, respectively) compared to all other groups (*P* < 0.001 and *P* = 0.012, respectively) (Figure 3B and C and Table 6).

In turn, overall median levels of CA 19-9 were 18.4 U/mL (IQR: 8.3-206) and did not differ statistically between groups: MCL: 20.4 U/mL (IQR: 14.0-35.8), HR: 16.9 U/mL (IQR: 11.7-18.1), NLOD: 18.8

Table 5 Biomarkers (glypican-1-positive circulating exosomes and carbohydrate antigen 19-9) profiles in the study groups, *n* = 88

Biomarker	MCL, <i>n</i> = 40	HR, <i>n</i> = 20	NLOD, <i>n</i> = 20	CG, <i>n</i> = 8	Total, <i>n</i> = 88	<i>P</i> value
GPC1 ⁺ crExos, %						< 0.001
Median	99.4	82.0	12.6	16.2	86.8	
Min/Max	6.4/99.9	1.1/99.0	2.1/93.9	1.2/24.5	1.1/99.9	
IQR	94.9-99.8	28.9-98.2	5.2-63.4	6.6-20.1	18.6-99.4	
CA 19-9 in U/mL						NS
Median	20.4	16.9	18.8	30.6	18.4	
Min/Max	8.5/206.6	8.3/28.0	12.8-48.7	13.7-69.6	8.3/206.6	
IQR	14.0-35.8	11.7-18.1	16.6-23.4	15.0-40.8	14.2-27.7	

P value for Kruskal-Wallis test. CA 19-9: Carbohydrate antigen 19-9; CG: Control group; HR: Hereditary risk; IQR: Interquartile range; GPC1⁺ crExos: Glypican-1 positive circulating exosomes; Max: Maximum; MCL: Mucinous cystic lesion; Min: Minimum; NLOD: New-late onset diabetes mellitus; NS: Non-significant.

Table 6 Size and concentration of exosomes according to the study groups, *n* = 88

	MCL, <i>n</i> = 40	HR, <i>n</i> = 20	NLOD, <i>n</i> = 20	CG, <i>n</i> = 8	Total, <i>n</i> = 88	<i>P</i> value
Size in nm						0.012
Median	82.5	94.7	100.7	72.8	90.5	
IQR	72.8-97.6	83.0-109.7	89.2-110.1	67.0-102.8	77.9-103.2	
Concentration as particles E + 10/mL						< 0.001
Median	3.48	3.36	6.05	3.30	4.10	
IQR	1.88-5.08	2.51-4.37	4.83-7.22	2.91-4.20	2.60-5.69	

P value for Kruskal-Wallis test. CG: Control group; HR: Hereditary risk; IQR: Interquartile range; MCL: Mucinous cystic lesion; NLOD: New-late onset diabetes mellitus.

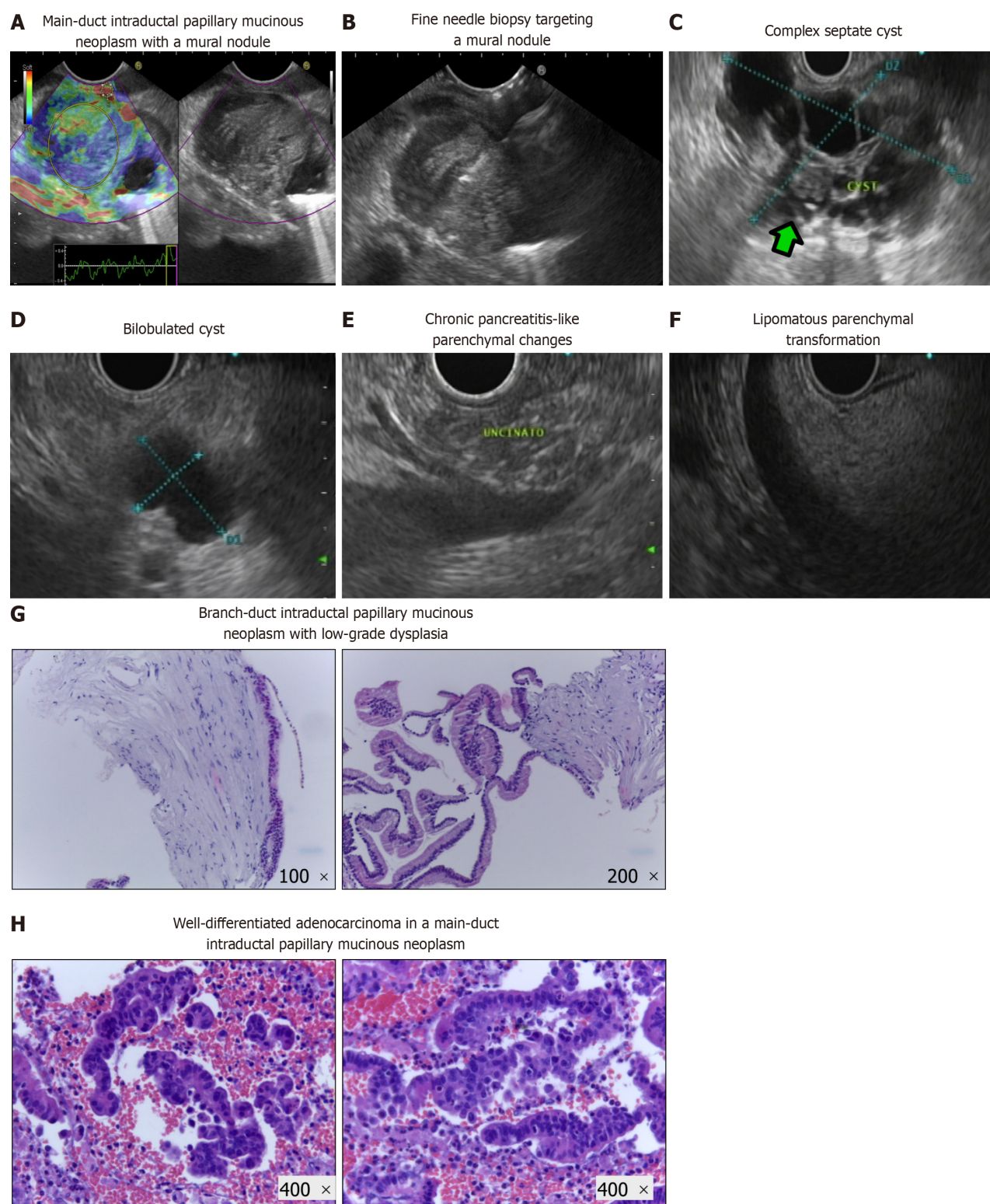
U/mL (IQR: 12.8-48.7) and CG: 30.6 U/mL (IQR: 13.7-69.6) (*P* = NS) (Figure 3D and Table 5). All CA 19-9 median levels were considered in the normal range considering the clinical standard cut-off (37 U/mL).

We further investigated the profile of both biomarkers in a sub-analysis in two distinct settings. First, among the HR group, we evaluated GPC1⁺ crExos and CA 19-9 levels according to the presence or absence of personal cancer history (Figure 4A and B). In the individuals with a positive history of cancer (5 colorectal and 1 cancer of the endometrium), although not statistically significant, we observed higher median levels of GPC1⁺ crExos (97.9%; IQR: 61.7%-99.5%) compared to those with a negative history (*n* = 14) (59.7%; IQR: 26.3%-96.4%) (*P* = 0.21). In relation to CA 19-9 median levels, they did not differ among these two subgroups (17.5 U/mL; IQR: 10.7%-20.1% and 16.3 U/mL; IQR: 12.2%-19.5%, respectively) (*P* = NS).

Second, in the total number of patients harboring pancreatic cystic lesions (40 in the MCL group, 2 diagnosed in the HR group, and 2 in the NLOD group), we studied the levels of GPC1⁺ crExos and CA 19-9 according to the presence or absence of WF/HRS in the EUS examination (Figure 4C and D). When WF or HRS were present (*n* = 20), patients presented with statistically significant higher median levels of GPC1⁺ crExos (99.6%; IQR: 97.6%-99.8%) compared to those with endosonographic "harmless" lesions (*n* = 24) (96.5%; IQR: 81.3%-99.5%) (*P* = 0.011) (Figure 4C). The levels of GPC1⁺ crExos presented an AUROC value of 0.723 in differentiating the group of cystic lesions with WF/HRS from those without (sensitivity 75.0% and specificity 67.7% for a cut-off of 98.5% (*P* = 0.012) (Figure 4E). CA 19-9 median levels did not statistically differ between these two subgroups (22.9 U/mL; IQR: 14.1-35.8 and 17.4 U/mL; IQR: 13.7-29.8, respectively) (*P* = NS) (Figure 4D).

DISCUSSION

MCLs are established as PDAC precursor lesions, with different risks considering morphologic



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Figure 2 Pancreatic abnormalities in endoscopy ultrasound examination and pathological exams of endoscopic ultrasound-guided fine needle aspiration/biopsy specimens from intraductal papillary mucinous neoplasms. A: Main-duct intraductal papillary mucinous neoplasm (MD-IPMN) with a large mural nodule, visualized in B-mode (right panel) and characterized with qualitative elastography (left panel) that revealed areas of hard consistency (blue color); B: Endoscopic ultrasound-guided fine needle biopsy with a 22 G needle targeting the harder lesions in the described mural nodule (pathological exam showed malignant degeneration); C: Complex septate cyst with areas of wall focal thickenings and a mural nodule (green arrow); D: Simple bilobulated cyst without worrisome features or high-risk stigmata; E: Chronic pancreatitis-like parenchymal changes (lobulation, hyperechoic foci, and strands); F: Homogenous hyperechoic appearance of pancreatic parenchyma suggestive of lipomatous transformation; G: Branch-duct IPMN with low-grade dysplasia [hematoxylin and eosin (H&E)-stained cellblock]. These images show the wall of a cystic lesion, partly lined by a cylindrical mucosecretory epithelium with low-grade atypia (left: 100 ×; right: 200 ×); H: Well-differentiated adenocarcinoma originated in a MD-IPMN (H&E-stained cellblock: 400 ×). These images show an epithelial neoplasm with marked architectural disorganization and severe cytological atypia. The cells have a high nucleus-cytoplasmic ratio, with anisokaryosis, irregular nuclei

with coarse chromatin and some with an exuberant nucleolus.

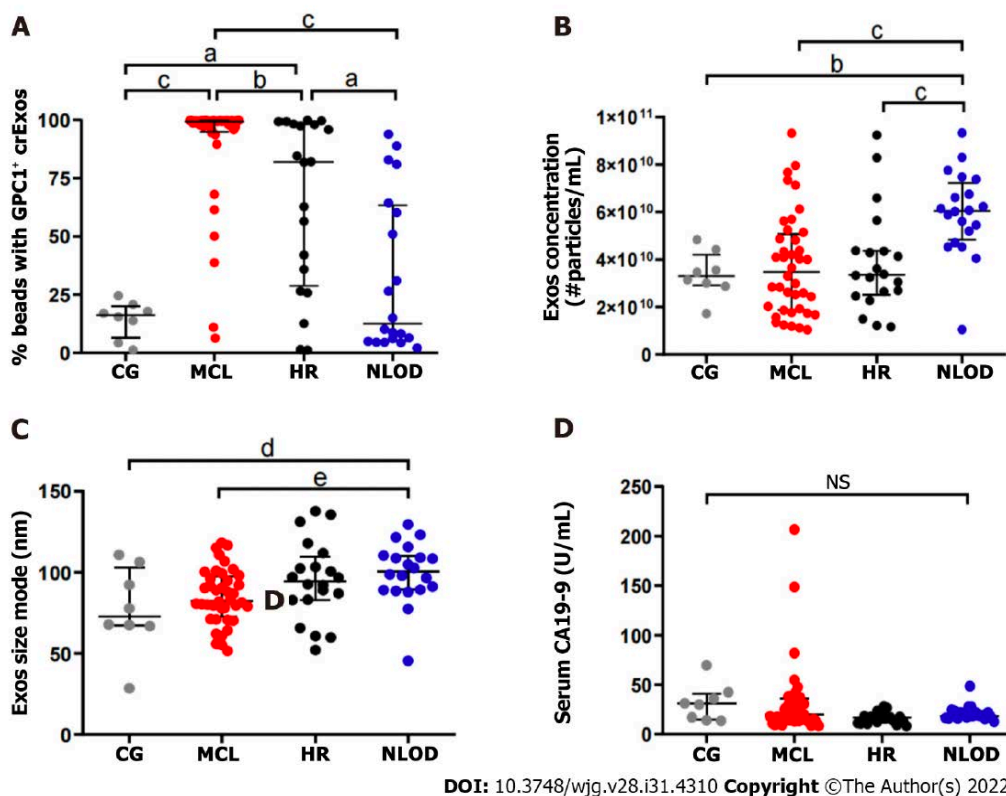
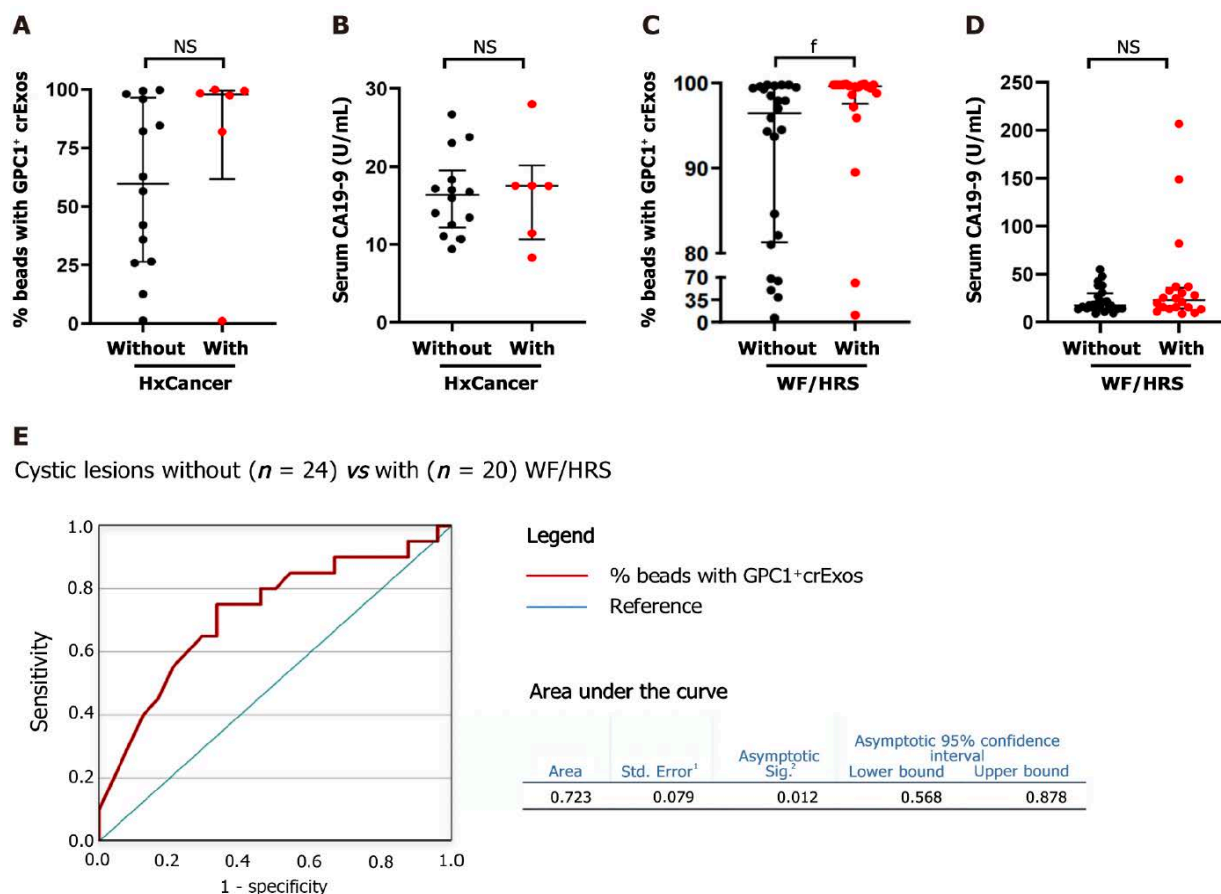


Figure 3 Glypican-1-positive circulating exosomes levels are different among the studied groups, whereas the levels of carbohydrate antigen 19-9 do not differ and are within the normal range in the entire population. A: Scatter dot plot representing the percentage of beads bound to glypican-1-positive exosomes (GPC1+ crExos) in the control group (CG) (n = 8), mucinous cystic lesions (MCLs) (n = 40), hereditary risk (HR) (n = 20) and new-late onset diabetes mellitus (NLOD) (n = 20), (Mann-Whitney U Test, ^aP < 0.02, ^bP = 0.005, ^cP < 0.001); B: Scatter dot plot representing exosomes concentration measured by nanoparticle tracking analysis depicting the number of exosomes/0.666 mL serum derived from CG (n = 8), MCL (n = 40), HR (n = 20), and NLOD (n = 20) donors (Mann-Whitney U Test, ^bP = 0.005, ^cP < 0.001); C: Scatter dot plot representing exosomes mode size distribution determined by nanoparticle tracking analysis from CG (n = 8), MCL (n = 40), HR (n = 20), and NLOD (n = 20) donors (Mann-Whitney U Test, ^dP = 0.018, ^eP = 0.04); D: Scatter dot plot representing the serum carbohydrate antigen 19-9 concentration (U/mL) as determined by enzyme-linked immunoassay in CG (n = 8), MCL (n = 40), HR (n = 20), and NLOD (n = 20), (Kruskal-Wallis Test, P = NS). Data are shown as the median ± interquartile range. NS: Non-significant; GPC1: Glypican-1; CA 19-9: Carbohydrate antigen 19-9; crExos: Circulating exosomes.

appearance as well as cytological findings resulting from guided tissue acquisition. While the diagnostic and management of pancreatic cysts differs between international guidelines[29,39-42], they all agree that the risk of malignancy should be based, essentially, in the assessment of the classical WF/HRS, which was initially defined in the Fukuoka consensus[43].

Our cohort mainly constituted IPMNs, most of which were BD-IPMN type and half of the lesions presented with WF and/or HRS[29]. Malignancy was suspected by EUS in two advanced lesions with main duct involvement, which were confirmed by cytology. Interestingly, the third confirmed malignant cyst corresponded to a case we have previously reported[38], and was diagnosed during a follow-up examination of an apparently “inoffensive” cystic lesion in the index EUS. Our results have confirmed the limited utility of the use of cytology alone for cyst etiological characterization[44,45], as 37% of the results of EUS-FNA of the cyst fluid were considered inconclusive by pathologists. This limitation of cyst fluid cytological analysis tends to be overwhelmed by the referral for surgical resection of indeterminate suspicious lesions. In fact, 15 of the 40 patients with MCL proposed for surgical treatment, after evaluation by a multidisciplinary team, corresponded to 3 cases with a positive cytology for malignancy and 12 patients with indeterminate/inconclusive results of EUS-FNA. The surgical specimen confirmed malignancy in all 3 cases with previous diagnosis and in the remaining 12 revealed benign IPMNs with low grade dysplasia. The overtreatment of some of these patients is an important issue, as the morbidity and mortality of pancreatic surgery is not negligible[46,47]. In the present cohort, 2 of the 5 deaths recorded were precisely due to complications after surgery, both in patients harboring benign lesions. This observation supports the drive of research, justifying the need to evaluate the role of upcoming biomarkers, like exosomal GPC1, in complement with EUS evaluation, for the stratification of MCL, ultimately contributing to refine the criteria for surgical treatment or continued surveillance. In



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Figure 4 Glypican-1-positive circulating exosomes are higher in individuals with personal history of cancer within the hereditary risk group, and among patients harboring pancreatic cystic lesions when worrisome features/high-risk stigmata are present. A: Scatter dot plot representing the percentage of beads bound to glypican-1 (GPC1)-positive circulating exosomes (GPC1⁺ crExos) in subjects in the hereditary risk (HR) group, without (n = 14) and with (n = 6) a personal history of cancer (HxCancer) [Mann-Whitney U Test, P = non-significant (NS)]; B: Scatter dot plot representing the serum carbohydrate antigen 19-9 (CA 19-9) concentration (U/mL) as determined by enzyme-linked immunoassay (ELISA) in subjects in the HR group, without (n = 14) and with (n = 6) HxCancer (Mann-Whitney U Test, P = 0.21); C: Scatter dot plot representing the percentage of GPC1⁺ crExos coupled to beads in subjects harboring pancreatic cystic lesions, without (n = 24) and with (n = 20) worrisome features (WF) or high-risk stigmata (HRS) (Mann-Whitney U Test, ¹P = 0.011); D: Scatter dot plot representing the serum CA 19-9 concentration (U/mL) as determined by ELISA in subjects harboring pancreatic cystic lesions, without (n = 24) and with (n = 20) WF/HRS (Mann-Whitney U Test, P = NS); E: Receiver operating characteristic curve of the cohort of pancreatic cystic lesions without (n = 24) or with (n = 20) WF/HRS, regarding the GPC1⁺ crExos levels (% beads with GPC1⁺ crExos). ¹Under nonparametric assumption; ²Null hypothesis: True area = 0.5. Data are shown as the median ± interquartile range. NS: Non-significant; GPC1: Glypican-1; crExos: Circulating exosomes; WF: Worrisome features; HRS: High-risk stigmata.

this setting, our preliminary results initially reported were promising, as the levels of GPC1⁺ crExos were equally elevated in patients with PDAC and with MCLs[48,49]. The findings in the present study confirm these observations, as the median levels of GPC1⁺ crExos observed in the MCL group (significantly higher than in the other studied groups) were in the same magnitude as the ones registered for PDAC patients, that we have recently reported using the same methodology[28]. Moreover, when we analyzed the levels GPC1⁺ crExos among all pancreatic cystic lesions (MCL group plus those detected in the screening EUS in HR and NLOD groups), we found that they were statistically more elevated when WF/HRS were present, suggesting that this biomarker can, in fact, have a role in the risk stratification of these PDAC precursor lesions.

This is the first study to access the profile of GPC1⁺ crExos in HR and NLOD individuals, and our results showed that the median levels of this biomarker were statistically more elevated among the HR group when compared to NLOD patients and controls. Considering our previous published observations[28], these levels of GPC1⁺ crExos in HR individuals were also higher than those registered among patients with CP (28.4%), but not as elevated as in those with PDAC (99.7%). Interestingly, our results also showed a tendency of the individuals with a history of previous cancer (mainly colorectal) to present higher levels of GPC1⁺ crExos when compared to those without. While PA were detected in only 10% among HR individuals, all representing simple cystic lesions, these observations may suggest a potential role of GPC1⁺ crExos as a marker of genetically determined predisposition for cancer development, even in the absence of “harmful” pancreatic lesions.

Important studies have recently been published[24-26] and prospective investigational projects are ongoing[50] demonstrating the importance to access the magnitude of the risk to develop PDAC among NLOD patients and to define PDAC incidence during long-term follow-up. This justified the inclusion of NLOD patients in our investigation cohort. We found PA in EUS in 35% of the NLOD patients, comprising simple cystic lesions, CP-like changes and lipomatous parenchymal transformations, all of them considered “harmless”, not requiring further investigation. Despite the highest median values of exosomes’ size and particle concentration being registered in this group, the percentage of crExos positive for GPC1 was low and not statistically different from the CG. This finding is in accordance with the lack of sufficient evidence, at the present, to include these patients in regular screening programs for PDAC early detection.

Some limitations of this study should be pointed out, namely the sample size, the possibility of a referral bias giving the nature of our tertiary center, the relatively short period of patients’ follow-up, and the cut-off found to stratify patients with cystic lesions and WF/HRS from those without. Also, the impossibility to include PanIN lesions in the study that would certainly power the analysis of GPC1 profile among PDAC pathological precursors. Lastly, the technique of exosomes isolation and analysis can still hamper its translation to clinical use. Despite these restraints, our results open the door for future studies to determine the value of this biomarker in the stratification of risk groups.

CONCLUSION

In summary, we demonstrate that GPC1⁺ crExos levels are elevated in MCL, in the same magnitude of PDAC patients. These levels were statistically higher in cysts harboring WF/HRS. High levels were also registered among individuals with HR for PDAC (predominantly in those with history of previous cancer). Longitudinal studies will clarify the potential of exosomal GPC1 as a biomarker for the diagnosis and stratification of PDAC precursor lesions, as well as in signaling individuals with genetic predisposition for this neoplasia, ultimately contributing to refine screening and surveillance strategies.

ARTICLE HIGHLIGHTS

Research background

Most of the patients with pancreatic ductal adenocarcinoma (PDAC) are diagnosed with advanced disease at which stage treatment is inefficient. In order to improve survival, new strategies to identify and stratify individuals at risk of developing PDAC [*i.e.* individuals with hereditary risk (HR), mucinous cystic lesions (MCLs) and new-late onset diabetes mellitus (NLOD)] are urgently needed. Endoscopic ultrasound (EUS) is one of the imaging modalities with better performance for the study of the pancreas. In turn, carbohydrate antigen 19-9 (CA 19-9), the only biomarker approved for clinical use, is still imperfect as it lacks sensitivity and specificity. Glypican-1 (GPC1) represents a promising candidate since it has been demonstrated to be overexpressed in circulating exosomes (crExos) of patients with PDAC, possibly allowing early detection.

Research motivation

To contribute to further evaluate the capacity of GPC1⁺ crExos to act as a potential biomarker for the investigation of individuals at risk for PDAC development, allowing for the early detection of the disease or its high-grade precursor lesions. Ultimately, we aim to contribute to improve patient’s survival.

Research objectives

We aimed to determine the capacity of GPC1⁺ crExos to identify individuals at higher risk to develop PDAC by comparing EUS pancreatic abnormalities (PA) with its levels within specific risk groups: HR, MCL and NLOD.

Research methods

We conducted a cross-sectional study with a prospective unicentric cohort including individuals with HR, MCL and NLOD, along with a control group (CG). All subjects were submitted to EUS at time of inclusion, with detailed characterization of detected PA. At the same time, blood samples were analyzed for GPC1⁺ crExos and CA 19-9 by flow cytometry and ELISA, respectively. EUS-guided tissue acquisition was performed whenever necessary. SPSS® 27.0 version was used for statistical analysis and all graphs were created using GraphPad Prism 7.

Research results

We found that: Individuals harboring MCL globally presented with the highest levels of GPC1⁺ crExos;

these levels were increased in the presence of worrisome features and or high-risk stigmata characterized by EUS; GPC1⁺ crExos levels were also elevated among individuals with HR for PDAC (predominantly in those with personal history of cancer), even in absence of harmful PA in EUS examination; and NLOD patients presented with GPC1⁺ crExos levels as low as those observed in the CG.

Research conclusions

Our study supports the role of GPC1⁺ crExos in the diagnosis and stratification of PDAC precursor lesions, namely MCL, and its eventual capacity in signaling individuals with genetic predisposition for this neoplasia, even when no harmful PA are detected by EUS.

Research perspectives

Our data encourage longitudinal studies to confirm the potential of exosomal GPC1 as a biomarker to be used in the future in the management of individuals at risk for PDAC.

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FOOTNOTES

Author contributions: Moutinho-Ribeiro P was the main project investigator, was responsible for patient selection and sample collection, performed pancreatic endoscopic ultrasound, and wrote the initial manuscript; Batista IA participated in the sample processing, supervised and collected the data, performed the data analyses, and supported the manuscript writing; Quintas ST participated in the sample processing, data collection and analyses, assembled the figures, and supported the manuscript writing; Adem B participated in the sample processing and supported the data analyses; Silva M participated in the data collection and performed the statistical analyses; Morais R, Peixoto A, Coelho R, Costa-Moreira P, and Medas R participated in patient observation, data collection, and database management; Medas R performed the statistical analyses; Lopes S and Vilas-Boas F performed the pancreatic endoscopic ultrasound; Baptista M, Dias-Silva D, Esteves AL, and Martins F participated in patient observation and data collection; Lopes J, Barroca H, and Carneiro F performed the cytological and histological examination of the pancreatic specimens; Macedo G conceived and designed the study, interpreted the data analyses, and critically reviewed the manuscript; Melo SA conceived and designed the study, supervised the data analyses, and critically reviewed the manuscript; All authors approved the final version to be published.

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Informed consent statement: All study participants or their legal guardian provided informed written consent about personal and medical data collection prior to study enrolment.

Conflict-of-interest statement: Sónia A Melo holds patents in the field of exosomes biology and are licensed to Codiak Biosciences, Inc. All other authors have no conflicts of interest to declare.

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3.5. DISCUSSION - THE CONTRIBUTION OF EUS AND BIOMARKERS:

3.5.1. In mucinous cystic lesions diagnosis & risk stratification

Pancreatic cysts are now a burden in global population health care, as their incidental detection has been increasing over time with the widespread use of sensitive and accurate abdominal cross-sectional imaging. Their overall prevalence is estimated to be 2.6–9.3% of asymptomatic patients undergoing abdominal MDCT and MRI scans, and they account for up to 20% of pancreatic resections in referral centers (328, 329).

Given the fact that MCLs, mainly IPMNs, constitute one of the most frequent and accessible PDAC precursor lesions, its proper diagnosis and risk assessment is crucial in pursuing the aim of changing the scenario of this dismal disease.

While most of pancreatic cystic lesions present a benign clinical course, others may already be high grade/invasive at diagnosis or, at least, harbor some risk for malignant transformation (330, 331). Among the later are the mucinous cysts (more frequently IPMNs and rarely MCNs), which definitive diagnosis and stratification according to potential malignant behavior may still be extremely challenging based on standard clinical parameters, imaging findings and ancillary studies (332, 333). This difficulty may result in an unbalanced cost-effective strategy in the management of patients.

For BD-IPMNs under long term surveillance, the risk of malignant transformation (cyst degeneration or concomitant carcinoma development) has recently been reported in a large cohort with over 1400 consecutive cases, with incidence rates of 3.3%, 6.6%, and 15.0% at 5, 10, and 15 years, respectively (82).

The main goal should be to accurately diagnose and resect high risk/early malignant lesions, while avoiding unnecessary surgery in low-risk ones, where continued follow up is indicated. For this purpose, highly sensitive and specific biomarkers, alone or in combination with imaging techniques, should be explored to improve effectiveness of upcoming screening and management strategies (334).

In terms of imaging modalities, MRCP and EUS assume a major role in cyst characterization and differential diagnosis. MRCP has the advantage of better evaluating the relation of the cystic lesion with the pancreatic ductal system, which if present, turns

the diagnosis of an IPMN the most likely. EUS adds a further dimension in the diagnosis of IPMNs and MCNs by improving the accuracy of the cyst wall and interior definition and allowing the possibility to perform FNA/B for fluid characterization and pathological exam (335, 336).

Within the past decade, focus has been put on the identification of biomarkers to accurately classify and stratify the risk of pancreatic cysts. DNA-based biomarkers, mostly related to KRAS and GNAS mutations, have demonstrated great potential and can be clinically available. Other promising biomarkers include miRNA profiling, differential mucin expression, inflammatory markers, and pancreatic cyst glucose testing (94). However, prospective studies are still lacking to demonstrate how these tests should be integrated in cyst evaluation algorithm.

For mucinous cysts differential diagnosis using the CEA fluid level > 192 ng/mL has remained the solely biomarker consistently applied for this purpose. Meanwhile, a recent metanalysis concluded that low pancreatic cyst fluid glucose (< 50 mg/dL) was associated with a high sensitivity and specificity with significantly improved diagnostic accuracy compared with CEA alone for the diagnosis of mucinous vs. nonmucinous pancreatic cystic lesions (92).

In the cohort of 40 patients included in our investigational project, for the diagnosis of MCLs we have used both imagiological and cyst content criteria (84). We have assumed IPMN aetiology when EUS and/or MRCP clearly showed communication of the cyst(s) with the pancreatic ductal system. In this setting, BD-IPMN were presumed preferentially if multiple and no dominant MPD dilation was documented. MD-IPMN was considered if a segmental or diffuse dilatation of the MPD > 5 mm was observed without any other cause of obstruction. Mixed-type IPMN was defined when features of MD-IPMN and BD-IPMN coexisted (104). EUS high risk-stigmata (HRS) and worrisome features (WF) were categorized according to the International Consensus of Fukuoka guidelines for the management of IPMN of the pancreas (104). A MPD dilatation ≥ 10 mm or an enhancing mural nodule ≥ 5 mm were classified as HRS, whereas a main duct dilatation between 5 and 9 mm, an enhancing mural nodule < 5 mm, a cyst diameter ≥ 3 cm, the presence of thickened/enhancing cyst walls, an abrupt change in the pancreatic duct caliber with distal pancreatic atrophy, and lymphadenopathy were considered WF (Table 2). Ideally, Doppler, elastography and CEUS image acquisition (sulphur

hexafluoride microbubbles - SonoVue[®], Bracco, Italy) were used for assistance in the characterization of mural nodules and thickened cyst walls.

Table 2. Classification of EUS high-risk stigmata and worrisome features of IPMN.

High-risk stigmata	Worrisome features
Enhancing mural nodule ≥ 5 mm	Enhancing mural nodule < 5 mm
MPD ≥ 10 mm	MPD 5-9 mm
	Thickened enhanced cyst wall
	Cyst size ≥ 3 cm
	Abrupt change in MPD with distal atrophy
	Lymphadenopathy

Adapted from Tanaka et al. (104).

We have performed EUS-FNA in almost all cystic lesions included, preferably with a 22G needle (Wilson-Cook[®] and Boston Scientific[®]), in a single pass, and along with antibiotic prophylaxis (single intravenous infusion of 200mg ciprofloxacin). Mural nodules or suspicious thickenings of the cyst wall or septa were punctured in a distinct pass, preferably with a 22G fine needle biopsy (FNB). Mucinous lesions were considered when fluid CEA was > 192 ng/mL and/or fluid glucose < 50 mg/dL. In relation to amylase content, if this was > 250 U/L a diagnosis of IPMN was likely, whereas levels < 250 U/L suggested an MCN. This last diagnosis was confirmed in typically non-communicant with the ductal system lesion, in which the pathological exam revealed a mucin-producing and septate cyst forming epithelial neoplasia with a distinctive ovarian-type stroma (77).

Our cohort was mainly constituted by IPMNs, most of them BD-IPMNs, with only 10% of cases classified as MD-IPMN or MT-IPMN. Half of the lesions were classified as presenting WF and/or HRS (104), mainly because of the EUS appearance (cyst size, presence of mural nodules, wall thickening and/or MPD dilation).

In this series, and although all specimens were evaluated by two experienced pathologists, the results of cytological exam was considered inconclusive in 37% of the procedures. In fact, although the role of EUS-FNA in pancreatic cystic lesions has evolved during the last decades, its results, in contrast with the high accuracy reported for solid ones, has not been as successful (88, 337). This aspect is predominantly justified by the difficulty in obtaining sufficient cells for diagnostic cytology, given the relatively low

cellularity of the aspirated fluid (338). Importantly, this aspect can have significant clinical consequences, as undetermined imagiological suspicious lesions are often referred for surgical resection. In our cohort, 15 of the 40 patients with MCLs proposed for surgical treatment, after evaluation by a multidisciplinary team, corresponded to 3 cases with a positive cytology for malignancy and to 12 patients with undetermined/inconclusive results of EUS-FNA. The surgical specimen confirmed malignancy in all 3 cases with previous diagnosis and in the remaining 12 revealed benign IPMNs with low grade of dysplasia, eventually representing cases where overtreatment might have occurred.

In order to improve the yield of pathological characterization, an advance in EUS-guided tissue acquisition was recently developed, consisting in performing a through-the-needle (19G) biopsy (TTNB) sampling of the lining of pancreatic cystic lesions with a dedicated microforceps (100-103). After relevant multicenter studies had demonstrated its feasibility, high diagnostic accuracy and clinical utility, our group started using TTNB sampling, especially in the presence of suspected BD-IPMNs with worrisome features (Fukuoka Consensus) (104) at EUS examination.

In fact, in another study carried out in our Department in which I have participated (data not included in this thesis), a prospective cohort evaluated the feasibility, diagnostic yield and safety of EUS-TTNB (339). Technical success was defined as visible specimen present after TTNB and clinical success was determined by the pathology results whenever the cyst wall lining was present, and a histologic diagnosis could be established. A total of 20 patients with a median cyst diameter of 29.5 mm were included. The procedure was technically successful in 19 patients and provided the diagnosis of cyst histotype in 15 cases (79%). In comparison, FNA cytology was performed in 19/20 patients but was nondiagnostic in 9 cases (45%). Cyst fluid CEA was > 192 ng/mL in only 5/20 (40%) of the cysts. TTNB allowed the definite diagnosis of 12 mucinous lesions, 10 of them BD-IPMN. Importantly, based on histological features and immunochemical mucin expression (MUC1, MUC2, MUC5AC and MUC6), we were able to determine the predominant phenotype in 8 of the IPMN cases. This aspect, in our opinion, besides cyst epithelium characterization, grade of atypia and specific diagnosis of histotype, should be included, whenever possible, in the pathological report, as the IPMN phenotype itself constitute an important parameter for risk stratification of the cyst and planning of the management (101). Nine patients were submitted to surgical resection and a diagnostic concordance of 66.7% was seen when compared with TTNB specimens.

Importantly, in 7 cases, TTNB provided the diagnosis otherwise not suggested by cytology, and in 8/20 patients (40%) it changed their management. As adverse events, only one self-limited intracystic bleeding was registered. We then concluded that EUS-TTNB was associated with high technical and clinical success and low adverse events rate. It was found superior to FNA cytology and cyst fluid CEA and its addition to the current standard approach may provide an incremental benefit for pancreatic cyst diagnosis and risk stratification.

Meanwhile, Kovacevic et al. (103), conducted a prospective, single-center, controlled study, to evaluate the added diagnostic value, clinical impact and safety of TTNB in 101 pancreatic cysts. Despite having demonstrated that the diagnostic yield of TTNBs for a specific cyst diagnosis was higher compared with FNA cytology (69.3% vs. 20.8%, respectively; $p < 0.001$), and resulted in a change of clinical management in about one in every 10 patients, the adverse event rate was 10%, with the most common event being acute pancreatitis. Importantly, 4 of the observed adverse events were severe, including one fatal outcome.

Taking in consideration this finding, we do think that caution must be taken when selecting patients where the benefits outweigh the risks, and that future safety studies are needed before including TTNB in the pancreatic cysts clinical algorithms.

As already mentioned in **chapter 3.2.1.**, several consensus and evidence-based guidelines, mostly based in clinical and EUS findings, have been developed by multiple medical societies (104-108) to assist clinicians in pancreatic cysts (mostly BD-IPMNs) management, importantly ruling its stratification in terms of malignant transformation and in selecting lesions to be surgically resected.

Meanwhile, none of these guidelines has gained unanimity worldwide, as validation studies have shown that they either have inadequate sensitivity (7.3%-35.2%) (340, 341) or inadequate specificity (23%-30%) (342).

Our group aimed to compare the accuracy of the International Consensus Guidelines (ICG) (104) with the American Gastroenterological Association (AGA) Guidelines (105) for the prediction of advanced IPMNs. In our cohort of 39 surgically resected BD-IPMNs (343), the sensitivity and specificity for advanced neoplasia was of 82% and 86% for AGA guidelines, and of 100% and 39% for ICG, respectively. We highlighted that both performances were suboptimal, as, in one hand, the higher specificity of AGA guidelines

was associated with a cost of lower sensitivity that could result in an important loss of some advanced lesions, and on the other hand, the lower specificity of ICG could be associated with a significant number of patients referred to unnecessary surgery.

In opposition to other guidelines, such as the ones proposed by the International Consensus (104, 335) and the European Consensus (108), that suggest a life-long closer follow-up of IPMN patients, one of the most controversial issues addressed in AGA guidelines (105) is the suggestion against continued surveillance if there has been no significant change in the characteristics of the cyst after 5 years of follow-up or if the patient is no longer a surgical candidate. Posterior studies doubted this measure as, in their cohorts, progression of certain “inoffensive” lesions was seen after that period of time.

Imbe et al. (344), in a validation study of these guidelines, analyzed data from 392 patients with IPMNs with at most one high-risk feature that were periodically followed up with imaging tests for more than 1 year (group 1) and 159 IPMN patients without worsening high-risk features after 5 years who stopped surveillance (group 2). In the first group, PC was identified in 12 patients (27.3%) when EUS-FNA was indicated vs. in none when it was not. In turn, in the “stop surveillance group”, cancer developed in three patients (1.9%) at 84, 103 and 145 months, respectively, and two of them died from it. Authors then concluded that although PC risk for IPMNs not showing significant change after 5 years is likely to be low, it is not negligible and suggested that the “stop surveillance” strategy should be reconsidered.

Crippa et al. (345) has also studied this issue and identified changes in 48% BD-IPMNs patients that were followed a median of 84 months. *De novo* worrisome features or high risk-stigmata (104) were observed in 26 patients (18%) after a median follow-up period of 71 and 77.5 months from diagnosis, respectively, without previous changes in 19 (73%) of them. These findings led the authors to advise that discontinuation of surveillance cannot be recommended and, instead, they propose intensification of surveillance after 5 years of diagnosis.

In an innovative recent study that included Dutch and American centers, with the aim to predict the risk of progression of low-risk BD-IPMN, specifically the development of WF/HRS over a period of 3 and 5 years, the authors proposed a stratification tool

(DART-I) that showed good performance, based on the score calculated considering initial cyst size and multifocality, patient's history of smoking, acute pancreatitis, and extra pancreatic malignancy (346).

In summary, at the present time, in many situations, there is still uncertainty in predicting the malignant potential of a designated lesion, impairing the choice of the more adequate management strategy. This aspect represents a truly global health problem, considering the high prevalence of asymptomatic patients harboring pancreatic cysts, the huge costs related with their surveillance, and, as importantly, the eventual morbimortality associated with surgical procedures.

For the above-mentioned reasons, efforts should be focused on the search of molecular markers that, together with specific clinical and EUS features, would contribute to clearly identify the small number of mucinous cystic lesions that harbor the highest risk of progression, in order to treat them before cancer occurs, the paramount to improve patient's life expectancy.

In this field of investigation, in 2014, Das et al. (111) described that a monoclonal antibody against a colonic epithelium phenotype (mAb Das-I) and reactive to premalignant conditions of the upper GI tract, was highly specific to tissue and cyst fluid from high-risk/malignant IPMNs. The same group (112) conducted a cross validation study in a multicenter cohort, including 169 pancreatic cystic lesions (90 IPMNs, 43 mucinous cystic neoplasms, and 36 non-mucinous cysts). Using histology findings from surgical samples as the reference standard, high-risk lesions were those with invasive carcinomas, high-grade dysplasia, or intestinal-type IPMNs with intermediate-grade dysplasia. An ELISA with mAb Das-I, performed in parallel using banked cyst fluid samples, was able to identify these high-risk lesions with 88% sensitivity and 98% specificity (95% accuracy), in a 10-fold cross validation analysis with 100 replications. In comparison, the International Consensus (104), and American Gastroenterological Association (105) guidelines criteria identified high-risk cysts with 52%, and 74% accuracy, respectively ($p < 0.001$). The authors, then, suggested that mAb Das-I might be used along with clinical guidelines to select patients at increased risk for cancer.

In turn, our group has focused on the study of GPC1 in PDAC as a biomarker for precursor lesions. In 2015, when Melo et al. (1) reported for the first time that GPC1

identifies cancer exosomes and detects early PC, in their discovery cohort of patients, 5 IPMN, all histologically validated, were included and classified as pancreatic cancer precursor lesions (PCPL). Despite this low number, the discovery cohort revealed that the levels of GPC1+ crExos distinguished patients with PCPL from healthy donors and patients with BPD. In detail, the levels of GPC1+ crExos in the PCPL group were consistently higher than the levels of GPC1+ crExos in the healthy donor group, as well as in the BPD group (constituted by 18 patients with CP and 8 with SCN). Of note, all patients in IPMN group revealed a macroscopic mass on MRI or MDCT imaging and presented with specific symptoms, findings that, in our opinion, may translate the presence of advanced lesions.

In 2018 we wrote a letter to the editor of *European Radiology* (110) in which we mentioned the preliminary data of our project combining EUS findings and this novel biomarker in patients diagnosed with PDAC or harboring a risk condition for it, namely: CP, family history of PDAC/associated hereditary syndrome, or a confirmed diagnosis of a cystic mucinous neoplasms (mainly IPMNs). Interestingly, at that time, in our initial cohort of 49 patients (113), we observed that the levels of GPC1+ crExos were significantly higher in patients with PDAC as well as in patients with IPMNs, when compared to the other mentioned risk groups. At that time, methodologically, we were using two distinct anti-GPC1 antibodies (Sigma-Aldrich® and Abnova®) for internal quality control. Levels of GPC1+ crExos were highly observed in both PDAC and IPMNs patients, independently of the antibody used, when compared to the other analyzed groups.

Interestingly, the detailed analysis of the mucinous cysts group revealed that the levels of GPC1+ crExos seemed to categorize it into two possible distinct subgroups of patients: those with GPC1+ crExos levels as high as PDAC patients and those with lower ones. We then questioned if this observation would constitute, in a near future, a true clue for stratifying pancreatic mucinous cysts according to its malignant potential that could enable an individualized management approach. This same nature of results was maintained when we analyzed our cohort extended to 110 patients, including 24 cases of MCLs (347).

Giving support to the idea that the levels of GPC1+ crExos might have a role in stratifying the risk of progression, while surveying our cohort of patients with mucinous cysts, we noted a very interesting and promising finding, that was published at the journal

Pancreatology (109). This was the case of a 58-year-old woman with history of breast cancer, in remission for 4 years, that was referred for the evaluation of a pancreatic cystic lesion, incidentally, discovered in follow-up imaging studies. At EUS, this was an apparently “inoffensive” 16 mm pancreatic cystic lesion, located at the body of the pancreas, communicating with the main pancreatic duct, that had normal caliber, suggesting a BD-IPMN. Despite no EUS suspicious findings were observed, but given the patient’s anxiety caused by the presence of a new potential neoplastic lesion, we decided to perform EUS-FNA for biochemical and cytological characterization of cystic fluid. Biochemistry was compatible with a mucinous origin (CEA – 662 ng/mL; amylase – 252 U/L), and cytology showed epithelial mucinous cells with low grade atypia (Papanicolaou Classification IVb.) (348). At that time, while no cancer had been diagnosed to this patient, she already presented an elevated percentage of GPC I + crExos (96.7%), parallel to those seen in PDAC individuals. A follow-up EUS-FNA was performed one year later, showing the persistence of the cystic lesion, with same morphologic characteristics. Surprisingly, high grade atypia was diagnosed in cytology, based on the presence of tridimensional epithelial groups with complex and disorganized architecture, composed of cylindric epithelial mucosecreting cells, with irregular large nucleus with membrane enhancement and nucleolus, along with mitotic figures (Papanicolaou Classification VI-malignant) (348). The pathological report upon a distal pancreatectomy concluded for a high-grade IPMN with biliopancreatic phenotype, and scattered lesions of PanIN of low grade (PanIN I and 2 subtypes) were also seen in the remaining parenchyma.

When we finally analyzed our entire cohort of 40 MCLs (84), we verified that the median levels of GPC I + crExos were 99.4%. This value is of the same magnitude of that we have described in a recent publication for PDAC patients (99.7%) (349).

Moreover, when we compared the subgroup of cysts with WF/HRS vs. those without (including 4 additional cystic lesions detected in screening EUS performed in hereditary risk individuals and in patients with NLOD), we found statistically higher levels in the formers (99.6% vs. 96.5%, respectively). For a cut-off of 98.5%, the levels of GPC I + crExos presented an area under the receiver operating characteristics (AUROC) value of 0.723 in differentiating the group of cystic lesions with WF/HRS from those without (sensitivity 75.0% and specificity 67.7%; $p = 0.012$).

Considering the clinical, pathological, and molecular data collected, we believe there is sufficient grounds to establish that the observed high values of GPC I + crExos possibly

correlated to the intrinsic potential for cytological progression, confirming the identity of MCLs as PDAC precursor lesions. Additionally, the pointed-out differences in values considering the presence or absence of WF/HRS features may contribute to further stratification of the malignant potential inherent to these lesions.

Ultimately, our results show the potential of introducing liquid biopsy and biomarkers as a promising clinical tool for the non-invasive assessment of PCPL, ultimately increasing the capacity to identify the patients that will benefit from surgical resection.

3.5.2. In discriminating PDAC from chronic pancreatitis

In their typical clinical and imagiological presentation, CP and PDAC represent two distinct entities, with a relatively easy differential diagnosis. Nevertheless, it should be highlighted that both diseases, besides sharing one of the most important risk factors, tobacco smoking, patients may present some common clinical signs, such as weight loss, abdominal pain and jaundice.

Additionally, CP is considered a risk condition for cancer development. In a recent metaanalysis (132), it was estimated that the risk of PDAC was elevated 16-fold, when the cases of cancers occurring in the first 2 years following a diagnosis of CP were excluded, in order to exclude initial misdiagnosis. Although a decline is seen over time, this risk persists still 8-fold increased when considering a 5-year lag period from CP to PDAC diagnosis.

Nevertheless, and when looking to the whole picture, only 5% of CP patients will eventually develop PDAC over a two-decade period (126), what, presently precludes the recognition of CP as a condition for general malignancy screening. Meanwhile, enrichment strategies to identify subset of individuals at very high-risk, the ones requiring special attention, would be more than welcomed.

Having said this, and in terms of differential diagnosis, in certain situations, the discrimination between both entities may be very challenging, especially if CP presents with a mass forming process, where the exclusion of malignant transformation is mandatory.

Given its superior spatial resolution, EUS is considered the most sensitive imaging technique for the diagnosis of CP, especially in its early stages, when other methods,

such as MDCT or MRI may not show any parenchymal or ductal abnormality. A total of 9 EUS criteria (4 parenchymal and 5 ductal) have been proposed by the International Working Group in the diagnosis of CP (Table 3) (350). Currently, although there is no optimal cut-off for establishing a EUS diagnosis of CP, the presence of 5 or more findings provides a definitive diagnosis of CP, whereas 2 or less findings effectively rule out this disease (351-355). These were the criteria we followed in our study (349).

Table 3. Standard EUS criteria for diagnosis of CP (International Working Group)

Parenchymal criteria	Ductal criteria
Hyperechoic foci	Main duct dilation
Hyperechoic strands	Duct irregularity
Lobular contour	Hyperechoic margins
Cysts	Visible side branches
	Stones

Adapted from Wallace et al. (350).

In general, EUS-guided tissue acquisition is considered the most reliable technique for detecting pancreatic malignancy, but in CP patients, namely in its mass forming presentation, the sensitivity decreases from 80-95% to 50-75% (353), with a reported false negative rate of 5-10%. Novel EUS imaging aids, such as elastography (E-EUS) and contrast-enhanced EUS (CEUS) have been proposed to increase the accuracy of EUS in the differential diagnosis between mass-forming (MF) CP and PC, especially directing EUS-guided tissue acquisition. Although theoretical strong, this evidence was only supported in unicentric studies, with inclusion of a low numbers of patients with CP (356, 357).

In turn, the current gold-standard biomarker used in the clinic, the carbohydrate antigen 19-9 (CA 19-9), has limitations concerning early PDAC detection. In addition to a high false negative rate, it lacks sensitivity and specificity in differentiating PDAC from CP, since 40% of the patients with CP can exhibit elevated CA 19-9 serum levels (216, 358). Hence, a biomarker that better discriminates PDAC from CP, specifically in its demanding MF presentation, would constitute a major improvement. In 2015, GPC I was put forward as a good candidate biomarker for early detection of PDAC, with distinct posterior studies showing that GPC I is overexpressed in circulating exosomes (crExos) of patients with PDAC when compared to healthy subjects or patients harboring BPDs

(1, 308, 310, 311, 315, 359, 360). Despite these findings, no study up to date has investigated the potential of GPC I+ crExos in a context of MF CP.

Bearing all these arguments in mind, especially the difficulty to exclude the presence of neoplastic changes in patients with CP, even after performing tissue acquisition guided by EUS, we have conducted a unicentric prospective cohort study with the aim to assess the accuracy of GPC I+ crExos to distinguish PDAC from CP, including its MF presentation, in comparison to the CA 19-9 biomarker. The findings of this study are reported in one of the original research papers of this thesis (349).

Our cohort was constituted by 60 PDAC and 29 CP patients (7 of them MF CP), all of them submitted to EUS. A separate blood sample collected at the same time was used to quantify CA 19-9 serum levels and assess of GPC I expression in circulating exosomes by flow cytometry and ELISA. EUS-guided tissue acquisition was performed in PDAC and in MF CP patients. All diagnostics were performed by two experienced pathologists having in consideration the clinical and imaging context of the patient. Samples were considered non-diagnostic if they did not meet the criteria of the Papanicolaou Society of Cytopathology System for Reporting Pancreaticobiliary Cytology (348), considering the patients' history, what was verified in 43% of the MF CP patients, even after repeating the biopsies. To exclude malignant transformation, particularly in MF presentation, the final diagnosis of CP was only determined after a minimum of 24 months follow-up period.

Considering that the differentiation between CP and PDAC is easier in advanced stages, when features of local invasion are usually present and support the diagnosis of malignancy, the usefulness of biomarkers would be more determinant to distinguish CP from early stage PDAC. We have also assessed the performance of CA 19-9 and GPC I+ crExos considering PDAC stages grouped in early (stage I) vs. advanced (stages II-IV) stages.

Our main findings were:

1. The percentage of GPC I+ crExos was significantly higher in PDAC (99.7%) in comparison with CP (28.4%; $p < 0.0001$), with an AUROC of 0.96 for a cut-off of 45% (98.3% sensitivity and 86.2% specificity $p < 0.0001$);
2. The percentage of GPC I+ crExos outperformed CA 19-9, with an AUROC of 0.82 for a cut-off of 37 U/mL (sensitivity 78.3% and specificity 65.5%; $p < 0.0001$);

3. The superiority of GPC1+ crExos over CA 19-9 in differentiating PDAC from CP was observed both in early (stage I) and advanced tumors (stages II-IV).
4. The median levels of GPC1+ crExos and CA 19-9 were not significantly different between the subgroups of MF and non-MF cases.
5. No differences between groups were obtained when the levels of GPC1+ crExos were determined using a commercially available ELISA kit (2.2% vs. 2.3%, $p = 0.161$).

Considering the levels of GPC1+ crExos coupled to beads, two statistical outliers were identified. One in the PDAC group which had a lower percentage (23.8%) in comparison to the mean of the group (99.7%). The other, in the CP group presented a higher level (99.9%) when compared to the median of the CP (28.4%). We found no malignant transformation during the surveillance of this CP patient. Nonetheless, during the 4th year of follow-up, a *de novo* 20 mm cystic lesion was detected by MDCT scan, and EUS-FNA was performed. The analysis was consistent with a BD-IPMN (amylase 12973 U/L, CEA 382 ng/mL, hypocellular cytology, with no epithelial cells), not harboring worrisome features but deserving further close monitoring. This observation may be interpreted as a false positive result, but the possibility of signaling a high-risk individual should not be discarded, as GPC1 has been shown to be equally elevated in some premalignant conditions such as MCLs (1, 84, 109).

If both the CP and PDAC outliers had been excluded from the analysis, for the same cut-off of 45.0%, the AUROC value would be 0.998 (sensitivity 100.0% and specificity 89.3%; $p < 0.0001$).

Another interesting finding was that one of the PDAC cases was initially recruited as a MF CP. However, upon the initial EUS, the biopsy indicated malignant transformation. Remarkably, the level of GPC1+ crExos coupled to beads was high (100.0%).

In sum, we validated the value of GPC1+ crExos coupled to beads to distinguish PDAC from CP with sensitivity and specificity superior to that of CA 19-9. This value was sustainable for both MF and non-MF presentation of CP, as well as for both early (stage I) and advanced (stages II-IV) tumors. Although the potential of crExos as

biomarkers in PDAC was previously demonstrated (1, 308, 311, 314, 315, 359-361), most of these studies compared PDAC with healthy individuals.

As also previously reported (1, 315), the fact that the results obtained using ELISA for GPC I+ crExos or serum GPC I do not reflect the data gathered from flow cytometry precludes its immediate translation to clinical practice. This can be explained, in part, by the fact that ELISA is able to detect free GPC I, independently of its ligation to exosomes. Therefore, a more technically feasible and cost-effective approach to be performed as routine analysis is necessary for this purpose.

Despite the current technical limitations, the potential clinical utility of GPC I+ crExos opens new opportunities in the differential diagnosis of PDAC in a context of CP.

3.5.3. In PDAC screening among individuals with hereditary risk and patients with new-late onset diabetes *mellitus*

As mentioned before, we have studied EUS findings and GPC I+ crExos profile in a cohort of patients diagnosed with PDAC and in subjects belonging to its associated risk groups. Part of the results from this investigation have already been discussed previously, specifically those related to MCLs diagnosis and risk stratification (84) and to the contribution in the differential diagnosis between PDAC and CP (349).

In this chapter, we will discuss the combination of EUS and GPC I, in the screening of PDAC among individuals with hereditary risk (HR) and patients diagnosed with NLOD. For this purpose, a cohort of these subjects was included in a prospective study, where we have accessed clinical data, EUS pancreatic findings and quantified the GPC I+ crExos levels. We aimed to evaluate the capacity of EUS and GPC I+ crExos to identify patients at higher risk to develop PDAC (84).

While at present only individuals harboring increased hereditary risk, either kindreds of familial pancreatic cancer (FPC) (7%) or belonging to other cancer syndromes with increased risk for PDAC (3%) are candidates for screening (57, 58, 67), there are other risk groups that should deserve special attention. In fact, these PDAC risk groups can constitute a refined population to be studied and surveilled in order to increase the rate of early detection and improve overall survival. One of this groups is constituted by the individuals older than 50 years with a recent (< 36 months) diagnosis of type II diabetes

mellitus (NLOD). In fact, some studies have pointed out that new onset DM can represent a clinical manifestation of asymptomatic PDAC or harbinger the disease (28, 119, 120, 122, 362). While the complex relationship between the two entities is yet to be fully understood, NLOD has been recognized as an entity signaling a 6- to 8- fold risk of developing PDAC in 3 years (116). Nevertheless, there are still no reliable biomarkers or imaging modalities able to identify early PDAC or to differentiate, in asymptomatic individuals, between the “common” type 2 DM of new onset and the PDAC-associated one (type 3c DM). This justifies, in part, the lack of recommendations in current guidelines for systematic PDAC screening among patients with NLOD.

It is generally accepted that individuals at increased risk may benefit from pancreatic cancer screening, essentially based on annual MRI or EUS (58, 67). For a screening program to be considered successful it should target the detection of early invasive tumors or, ideally, its advanced precursor lesions, i.e., mucinous cystic or PanIN lesions with high grade dysplasia, that can be treated by curative surgical resection (51). In general, these types of lesions are identified in 2% to 5% of all high-risk individuals undergoing screening programs (195, 196).

In relation to the best imaging method, several studies have pointed out EUS as having better accuracy for the detection of small (< 2 cm) solid lesions, while MRI, specially MRCP, being superior in characterization of small cysts, namely due to its ability to demonstrate its relation to the pancreatic ductal system (173, 197, 198).

In our investigational cohort, for PDAC screening purposes, we have included 20 individuals with hereditary risk (HR) for PDAC and 20 patients diagnosed with NLOD. A control group was also included that consisted of individuals submitted to EUS for other reasons than pancreatic pathology, with a normal pancreas and absence of hereditary/familial risk factors.

The HR group included 2 kindreds of FPC, 1 case of PJS and 17 cases of Lynch syndrome. All these individuals had a clinically and genetically established diagnosis and have been followed in a dedicated consultation for hereditary digestive cancers in our Institution. Sixty-five percent had family history of PDAC in at least one FDR. Of note, 35% of the Lynch syndrome individuals had a personal history of past cancer (5 colorectal and 1 endometrial), all disease-free at the time of inclusion.

In the NLOD group we have included patients aged 50 or more years that have been diagnosed with type II DM within a period of 36 months. The diagnosis of type 2 diabetes mellitus was made according the American Diabetes Association criteria (363).

Among HR group, EUS detected PA in 10% of the individuals, all consisting in simple cystic lesions, without HRS/WF. This finding is in accordance with most studies, reporting the identification of these lesions in 13%-20% among these subjects, more frequently than in the general population (1%-5%) (173, 200). While in a recent study by Canto et al. (24), reporting their experience following more than 350 HR individuals, a cumulative detection rate of 7% for high-risk lesions (median follow-up of > 5 years) and an annual rate of progression of 1.6% were described, in our cohort, no progression or cancer development was observed in this group of individuals during a median follow-up of 35 months.

In turn, in NLOD group, EUS found PA in 35% of patients, comprising simple cystic lesions, CP-like parenchymal changes and lipomatous parenchymal transformations. All these PA were considered “harmless”, not requiring further investigation, namely EUS-guided FNA/B. While cysts and CP-like changes are frequently described in studies evaluating HR individuals (24, 197, 212), lipomatous parenchymal transformation is not. This represents an entity with unclear aetiology that has been attributed to several metabolic disturbances commonly found in diabetic patients (364). The fact that in our cohort, NLOD patients were the ones presenting the highest body mass index (29.9 ± 4.5 kg/m²) may also explain why this PA was uniquely found among this group.

The inefficiency of CA 19-9 as a biomarker for early diagnosis and lesion’s risk assessment has been fueling the need for investigation of upcoming molecules for this purpose. In the present cohort, not surprisingly, the median levels of CA 19-9 were normal and not statically different among the considered groups.

Our work is pioneer in accessing the profile of GPC I in circulating exosomes in HR individuals and patients diagnosed with NLOD. Our data showed that the median levels of GPC I were statistically more elevated among the HR group (82.0%) when compared to controls and NLOD patients (16.2% and 12.6%, respectively). Considering the findings reported in a previous study by our group using the same methodology (349), the levels of GPC I+ crExos registered in HR individuals were also higher than those verified

among patients with CP (28.4%), but not as elevated as in PDAC ones (99.7%). Moreover, and interestingly, among the HR group, our results also showed a tendency of the individuals with a history of previous cancer (mainly colorectal) to present higher levels of GPC I + crExos (97.9%) when compared to those without (59.7%). In contrast to PDAC and breast cancer, where evidence has shown the overexpression of GPC I in these neoplasias (1, 305), controversies exist in relation to colorectal cancer. While Fernández-Vega et al. (301) reported that both GPC I mRNA and protein expression levels were increased in colorectal cancer, De Robertis et al. (365) found them decreased, even in metastatic disease. Nonetheless, and taken together these observations, we may hypothesize that GPC I may function as a marker of a genetically determined predisposition for cancer development, even in the absence of harmful PA in EUS examination. This will certainly open a path for future investigations. At the same time, it will be also motivating to verify, in a longitudinal study, what will be the changing profile of GPC I + crExos among this HR population. Hopefully, overtaken some technical restrains that exist at the present (84, 349), this molecule can have the potential to be considered, in the future, to have a role acting both as a diagnostic and surveillance biomarker.

In relation to NLOD patients, in our study, nor the findings in EUS nor the levels of GPC I + crExos, that were equal to controls, can support the inclusion of this group of subjects in dedicated screening programs. In fact, and since only a small proportion of NLOD patients will develop sporadic PDAC, at the present time, it should be taken in consideration enrichment strategies to identify the subset of individuals at very high-risk, before using them as a potential group for screening for early PDAC detection.

3.6. FINAL REMARKS AND FUTURE DIRECTIONS

This PhD thesis brought comprehensive data on the combination of the use of a gold standard imaging method, EUS, along with GPC I, a very promising biomarker, in the clinical management of PDAC patients and its associated groups of risk.

Importantly, we have demonstrated that:

1. PDAC patients presented high levels of GPC I + crExos.
2. The levels of GPC I were also high among patients with pancreatic MCLs, parallel to those observed in PDAC patients, suggesting that this biomarker can potentially be used in the detection of PDAC precursor lesions.
3. Among MCLs, higher levels of GPC I were seen in those presenting worrisome features/high risk stigmata in the EUS evaluation. This evidence may represent an ability to further assist in PDAC precursor lesions risk stratification, contributing to the better selection of patients suitable for surveillance vs. prompt therapeutical intervention.
4. GPC I, as a complement to EUS, had the capacity to discriminate PDAC from CP. Most importantly, it gains special relevance in some demanding mass-forming presentations of this inflammatory condition.
5. The capacity of GPC I in discriminating PDAC from CP was superior to that observed for CA 19-9 and was validated both for early (stage I) and advanced tumors (stages II-IV).
6. When performed for screening purpose, EUS revealed pancreatic abnormalities in 10% of the individuals with hereditary risk and in 35% of the patients diagnosed with NLOD, all of them considered echoendoscopically “harmless”.
7. Although not as elevated as in PDAC and MCLs patients, GPC I levels were also high among individuals with hereditary risk (predominantly in those with history of previous cancer), even in the absence of harmful EUS pancreatic lesions. This suggests a possible role of this biomarker in signaling genetic predisposition for malignancy, supporting the pertinence of ongoing screening program indicated for these individuals.

8. On the contrary, the levels of GPC I were low among patients with NLOD (parallel to those registered in the control group), not favoring, at the present time, their inclusion in dedicated PDAC screening programs.

Regarding the future perspectives in light of our results, continuing the long-term follow-up of our cohort, along with inclusion of new cases, will allow us to strength the insights already achieved and provide new scientific evidence.

It will be very interesting to observe the changes in the profile of GPC I levels, along with the clinical evolution of the detected and eventually *de novo* pancreatic abnormalities, among the studied groups with increased risk for PDAC. In particular, the continued EUS surveillance of MCLs will allow, in the future, to access the accuracy of the initial GPC I levels in the risk stratification of these PDAC precursor lesions.

Research should continue to improve the capacity of imaging methods in the detection and characterization of pancreatic minimal changes. Among them, are the PanIN lesions, that constitute, along with MCLs, the established PDAC precursor lesions. In fact, one of the major limitations is that current imaging modalities fail to detect these microscopic neoplastic changes.

Future investigation should focus on the role of molecular target-imaging for this purpose. Specifically, the use of known or upcoming biomarkers as contrast agents could increase the accuracy of imaging detection, aiming at the identification of very small early tumors and high-risk precursor lesions.

Another exciting field of research is that of imaging diagnosis related to the use of artificial intelligence-based tools. In this area, our group has already started a project with the aim to better diagnose and categorize pancreatic cystic lesions by artificial intelligence assisted EUS. We do think that this technology, in the future, could have applications in other imaging methods aiming different types of pancreatic lesions, so that it will enhance the detection capacity of lesions below the threshold of current clinical gold-standard modalities.

The current technical limitations in quantifying exosomal GPC I, may jeopardize its possible translation to clinical use. Several alternative strategies are under development, including the use of microfluidics to detect GPC I in exosomes isolated from patient

samples. The development of nanomedicine and nanodevices opens the door for the possibility to get GPC I into the clinic in the future.

Meanwhile, resources should be directed to allow research in order to increase our knowledge on the behavior of this neoplasia, specially at the cellular and microenvironmental level. These discoveries will allow the development of new target therapies, that will definitely contribute to change the current dark scenario of most PDAC patients.

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4. APPENDIX

In this section, other PhD-related outputs are listed (peer-reviewed papers, case reports, letters to editor, book chapter and published abstracts in indexed journals), as some of its contents are also referred in the Discussion chapter of the thesis.

4.1. OTHER PhD-RELATED OUTPUTS

4.1.1. Peer-reviewed papers

1. Moutinho-Ribeiro P, Peixoto A, Macedo G. **Endoscopic retrograde cholangiopancreatography and endoscopic ultrasound: To be one traveler in converging roads.** GE Port J Gastroenterol. 2018 Apr;25(3):138-145. DOI: 10.1159/000481537. Epub 2017 Oct 25.
2. Moutinho-Ribeiro P, Costa-Moreira P, Caldeira C, Leite S, Marques S, Moreira T, Nunes N, Bispo M. **GRUPUGE Perspective - Endoscopic ultrasound-guided celiac plexus interventions.** GE Port J Gastroenterol 2020; 28:32-38. DOI: 10.1159/000508293.
3. Bispo M, Caldeira C, Leite S, Marques S, Moreira T, Moutinho-Ribeiro P, Nunes N. **GRUPUGE Perspective - Endoscopic ultrasound-guided ablation of focal pancreatic lesions.** GE Port J Gastroenterol 2020; 27:410–416. DOI: 10.1159/000507895.
4. Marques S, Bispo M, Leite S, Moreira T, Caldeira C, Moutinho-Ribeiro P, Nunes N. **GRUPUGE Perspective - Endoscopic ultrasound-guided fine-needle tattooing and fiducial placement in pancreatic cancer.** GE Port J Gastroenterol 2020; 28:106–110. DOI: 10.1159/000509194.
5. Costa-Moreira P, Vilas-Boas F, Martins D, Moutinho-Ribeiro P, Lopes S, Lopes J, Barroca H, Macedo G. **Use of suction during endoscopic ultrasound-guided fine needle biopsy of solid pancreatic lesions with a Franseen-tip needle: a pilot comparative trial.** Endosc Int Open. 2021 Mar; 9(3): E401–E408. DOI: 10.1055/a-1336-3170.

6. Manuel Cruz, Pedro Moutinho-Ribeiro, Pedro Costa-Moreira, Guilherme Macedo. **Solid pseudopapillary neoplasm of the pancreas: unfolding an intriguing condition.** GE Portuguese Journal of Gastroenterology 2021. DOI: 10.1159/000519933.
7. Carolina F. Ruivo, Nuno Bastos, Ines A. Batista, Barbara Adem, Cecília Duraes, Carlos A. Melo, Francisco Campos-Laborie, Pedro Moutinho-Ribeiro, Barbara Morão, Ana Costa-Pinto, Soraia Silva, Hugo Osorio, Sergio Ciordia, Jose Luis Costa, David Goodrich, Bruno Cavadas, Luisa Pereira, Tony Kouzarides, Guilherme Macedo, Rui Maio, Fatima Carneiro, Marília Cravo, Raghu Kalluri, and Jose C. Machado, Sonia Melo. **Extracellular Vesicles from Pancreatic Cancer Stem Cells Lead an Intratumor Communication Network (EVNet) to Fuel Tumor Progression.** Gut. 2022 Jan 10:gutjnl-2021-324994. DOI: 10.1136/gutjnl-2021-324994. PMID: 35012996.
8. Filipe Vilas-Boas, Tiago Ribeiro, Pedro Costa-Moreira, Helena Barroca, Joanne Lopes, Diana Martins, Pedro Moutinho-Ribeiro, Guilherme Macedo. **Endoscopic ultrasound through-the-needle biopsy of pancreatic cysts: towards procedure standardization.** (Accepted for publication in Dig Dis. 2022).

4.1.2. Case reports

1. Peixoto A, Moutinho-Ribeiro P, Macedo G. **Degenerate intraductal papillary mucinous carcinoma with fish-mouth appearance of the papilla.** J Gastrointest Liver Dis. 2017 Jun;26(2):113. DOI: 10.15403/jgld.2014.1121.262.
2. Carmo J, Moutinho-Ribeiro P, Bispo M, Chagas C. **Elastography and contrast-enhanced endoscopic ultrasound findings in a pseudo-solid variant of a pancreatic serous cystadenoma.** GE Port J Gastroenterol. 2017 Sep;24(5):252-254. DOI: 10.1159/000478941.
3. Medas R, Moutinho-Ribeiro P, Morais R, Marques A, Barroca H, Macedo G. **One time shop EUS: Unravelling two synchronous tumors.** (Submitted to Endosc Ultrasound).

4.1.3. Letters to editor

1. Costa-Moreira P, Vilas-Boas F, Moutinho-Ribeiro P, Macedo G. **Endoscopic ultrasound FNB and macroscopic on-site evaluation (MOSE) with combined cytological and histological analysis may overcome the need of rapid on-site evaluation (ROSE).** *Endosc Ultrasound*. 2019 Nov-Dec; 8(6): 432–433. DOI: 10.4103/eus.eus_72_19.
2. Vilas Boas F, Moreira P, Moutinho-Ribeiro P, Martins D, Lopes J, Macedo G. **Through-the-needle biopsy sampling may allow preoperative intraductal papillary mucinous neoplasia subtyping.** *Gastrointest Endosc*. 2020; 92 (1): 235-237. DOI: 10.1016/j.gie.2020.02.037.

4.1.4. Book chapter

1. Ferreira da Silva J, Moutinho-Ribeiro P, Rodrigues-Pinto E. **Diagnóstico precoce e rastreio.** In: **Cancro do Pâncreas – Atualizações.** *Castelo Branco: Edição Club Português do Pâncreas, 2020. p.45-58.*

4.1.5. Published abstracts (indexed journals)

1. Coelho R, Moutinho-Ribeiro P, Gaspar R, Andrade P, Liberal R, Morais R, Costa-Moreira P, Santos AL, Vilas-Boas F, Lopes S, Pereira P, Macedo G. **Resectable pancreatic cancer: are we performing a good pre-operative staging?** (*United European Gastroenterology Week 2016. Viena, Austria, 15-19 October 2016*). *United European Gastroenterol J*. 2016; 2 (Supplement 1).
2. Gaspar R, Moutinho-Ribeiro P, Andrade P, Coelho R, Silva M, Peixoto A, Liberal R, Morais R, Santos A, Costa-Moreira P, Macedo G. **22. Pancreatic carcinoma: still a gloomy reality.** (*American College of Gastroenterology Annual Meeting 2016. Las Vegas, EUA, 17-19 October 2016*). *Am J Gastroenterol*. October 2016; 111:S1223-S1224.

3. Silva M, Coelho R, Moutinho-Ribeiro P, Gaspar R, Andrade P, Costa-Moreira P, Santos LA, Liberal R, Morais R, Vilas-Boas F, Lopes S, Pereira P, Macedo G **Pancreatic intraepithelial neoplasia (PanIN) in resected pancreatic cancer.** (49th Meeting of European Pancreatic Club. Budapest, Hungary, 28 June-1 July 2017). *Pancreatology*, Volume 17, Issue 3, Supplement, July 2017, Page s85.
4. Gaspar R, Andrade P, Moutinho-Ribeiro P, Coelho R, Liberal R, Morais R, Costa Moreira P, Santos A, Vilas-Boas F, Lopes S, Macedo G. **Diagnostic performance and factors influencing the accuracy of EUS-FNA for pancreatic adenocarcinoma.** (49th Meeting of European Pancreatic Club. Budapest, Hungary, 28 June-1 July 2017). *Pancreatology*, Volume 17, Issue 3, Supplement, July 2017, Pages s81-s8.
5. Vilas-Boas F, Moutinho-Ribeiro P, Lopes S, Rodrigues-Pinto E, Pereira P, Bouça-Machado T, Costa-Maia J, Macedo G. **International consensus guidelines and American Gastroenterological Association guidelines for the prediction of advanced pancreatic intraductal papillary mucinous neoplasms (IPMNs).** (49th Meeting of European Pancreatic Club. Budapest, Hungary, 28 June-1 July 2017). *Pancreatology*, Volume 17, Issue 3, Supplement, July 2017, Pages s95-s96.
6. Moutinho-Ribeiro P, Silva S, Adem B, Silva M, Lopes S, Vilas-Boas F, Melo AS, Macedo G. **Glypican-I circulating exosomes levels are higher in pancreatic adenocarcinoma and cystic mucinous neoplasms than in other associated risk groups for pancreatic cancer: clues for early diagnosis?** (49th Meeting of European Pancreatic Club. Budapest, Hungary, 28 June-1 July 2017). *Pancreatology*, Volume 17, Issue 4, Supplement, July 2017, Pages s63-s64.
7. Moutinho-Ribeiro P, Silva S, Adem B, Silva M, Coelho R, Lopes Susana, Vilas-Boas F, Barroca H, Machado J, Carneiro F, Melo S, Macedo G. **Exosomal glypican-I in patients with pancreatic adenocarcinoma and associated risk groups.** (World Congress of Gastroenterology at American College of Gastroenterology 2017. Orlando, EUA, 13-18 October 2017). *Am J Gastroenterol*. October 2017; 112:S10.

8. Gaspar R, Moutinho-Ribeiro P, Magno-Pereira V, Silva M, Peixoto A, Lopes S, Vilas-Boas F, Barroca H, Lopes J, Carneiro F, Macedo G. **Rapid on-site evaluation (ROSE): an essential tool in echo-endoscopic (EUS) study of solid lesions of the pancreas.** (*United European Gastroenterology Week 2017. Barcelona, Spain, 28 October- 1 November de 2017*). *United European Gastroenterol J.* 2017; 5 (Supplement 1).

9. Moutinho-Ribeiro P, Silva M, Batista I, Adem B, Silva S, Morais R, Coelho R, Lopes S, Vilas-Boas F, Baptista M, Barroca H, Machado JM, Carneiro F, Melo SA, Macedo G. **Exosomal glypican-1 and pancreatic adenocarcinoma: prime time for early diagnosis?** (*World Congress of Gastroenterology at American College of Gastroenterology 2018. Philadelphia, EUA, 5-10 October 2018*). *Am J Gastroenterol.* October 2018; 113:S44.

10. Pereira VM, Moutinho-Ribeiro P, Vilas-Boas F, Morais R, Lopes J, Portugal R, Amendoeira I, Macedo G. **Pancreatic atypical neuroendocrine tumor as a mimicker of a pseudo-papillary solid neoplasia.** (*United European Gastroenterology Week 2018. Vienna, Austria, 20-24 October 2018*). *United European Gastroenterol J.* 2018; 6 (Supplement 1).

11. Gaspar R, Andrade A, Moutinho-Ribeiro P, Vilas Boas F, Lopes S, Barroca H, Lopes J, Carneiro F, Macedo G. **Impact of inconclusive or negative endoscopic ultrasound-guided fine-needle aspiration results in the management of suspicious solid pancreatic masses.** (*United European Gastroenterology Week 2018. Vienna, Austria, 20-24 October 2018*). *United European Gastroenterol J.* 2018; 6 (Supplement 1).

12. Costa Moreira P, Vilas-Boas F, Moutinho-Ribeiro P, Pereira P, Lopes S, Rodrigues Pinto E, Macedo G. **Diagnostic yield of international guidelines on predicting advanced histology in branch duct pancreatic intraductal mucinous neoplasms (BD-IPMN) of the pancreas: a real-life study.** (*United European Gastroenterology Week 2019. Barcelona, Spain, 19-23 October 2019*). *United European Gastroenterol J.* 2019; Vol. 7 Issue 8 (Supplement).

13. Costa Moreira P, Vilas-Boas F, Carvão J, Gaspar R, Pereira V, Peixoto A, Silva M, Moutinho-Ribeiro P, Lopes S, Macedo G. **Endoscopic ultrasound fine-needle biopsy and macroscopic on-site evaluation (MOSE) with combined cytological and histological analysis may overcome the need of rapid on-site evaluation (ROSE): a prospective study.** (*United European Gastroenterology Week 2019. Barcelona, Spain, 19-23 October 2019*). *United European Gastroenterol J.* 2019; Vol. 7 Issue 8 (Supplement).

14. Moutinho-Ribeiro P, Moreira P, Morais R, Vilas-Boas F, Lopes S, Macedo G. **Endoscopic ultrasound in the diagnosis and staging of pancreatic cancer: a comparison with computer tomography imaging.** (*American College of Gastroenterology Annual Meeting 2019. San Antonio, EUA, 25-30 October 2019*). *Am J Gastroenterol.* October 2019; 114:S8.

15. Moutinho-Ribeiro P, Morais R, Costa-Moreira P, Baptista M, Dias-da-Silva D, Vilas-Boas F, Lopes S, Macedo G. **Endoscopic ultrasound for screening patients with increased risk of pancreatic ductal adenocarcinoma: miles to go before we sleep.** (*American College of Gastroenterology Annual Meeting 2019. San Antonio, EUA, 25-30 October 2019*). *Am J Gastroenterol.* October 2019; 114:S7-S8.

16. Costa-Moreira P, Vilas-Boas F, Moutinho-Ribeiro P, Martins D, Lopes J, Macedo G. **Technical feasibility and diagnostic yield of EUS- guided through-the-needle biopsy forceps for pancreatic cystic lesions - experience of a tertiary referral center.** (*American College of Gastroenterology Annual Meeting 2020. Digital event, EUA 23-28 October 2020*). *Am J Gastroenterol.* October 2020, 115: S41.

17. Costa-Moreira P, Vilas-Boas F, Martins D, Moutinho-Ribeiro P, Lopes S, Lopes J, Barroca H, Carneiro F, Macedo G. **The use of suction during endoscopic ultrasound-guided fine needle biopsy of solid pancreatic lesions using Franseen-tip needle: a randomized crossover trial.** (*United European Gastroenterology Week 2020. Digital event, 10-13 October 2020*). *United European Gastroenterol J.* October 2020; 8 (Supplement): p804

Porto, 2022

A handwritten signature in black ink, reading "Pedro Moutinho Ribeiro". The signature is written in a cursive, flowing style with a large initial 'P' and a long, sweeping underline.

Pedro Moutinho Ribeiro

FACULDADE DE MEDICINA

