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Recent developments in the diagnosis of pancreatic neuroendocrine neoplasms

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ABSTRACT

Introduction: Pancreatic Neuroendocrine Neoplasms (PanNENs) are characterized by a highly heterogeneous clinical and biological behavior, making their diagnosis challenging. PanNENs diagnostic work-up mainly relies on biochemical markers, pathological examination, and imaging evaluation. The latter including radiological imaging (i.e., computed tomography [CT] and magnetic resonance imaging [MRI]), functional imaging (i.e., 68Gallium [68Ga]Ga-DOTA-peptide PET/CT and Fluorine-18 fluorodeoxyglucose [18F]FDG PET/CT), and endoscopic ultrasound (EUS) with its associated procedures.

Areas covered: This review provides a comprehensive assessment of the recent advancements in the PanNENs diagnostic field. PubMed and Embase databases were used for the research, performed from inception to October 2023.

Expert opinion: A deeper understanding of PanNENs biology, recent technological improvements in imaging modalities, as well as progresses achieved in molecular and cytological assays, are fundamental players for the achievement of early diagnosis and enhanced preoperative characterization of PanNENs. A multimodal diagnostic approach is required for a thorough disease assessment.

Article Highlights

- PanNENs are characterized by a highly heterogeneous biological behavior and a delayed diagnosis is frequently observed.
- Diagnostic modalities advances have allowed an early and accurate PanNENs identification.
- Significant progress has been made in noninvasive preoperative characterization of PanNENs.
- Multimodal diagnostic approach is recommended for a comprehensive PanNENs evaluation.

1. Introduction

Pancreatic neuroendocrine neoplasms (PanNENs) comprise a highly heterogeneous group of lesions arising from neuroendocrine cells of the islet of Langerhans. PanNENs are characterized by the expression of neuroendocrine markers (i.e., chromogranin A, synaptophysin, somatostatin receptors) [1,2]. Moreover, PanNEN cells overexpress a plethora of proangiogenic factors (i.e., vascular endothelial growth factor, platelet-derived growth factor, angiopoietins), resulting in hypervascular lesions [3]. Indeed, the presence of a dense vascular network is a typical feature of PanNENs, that frequently allows their recognition at imaging studies [3,4]. Notably, a negative correlation between PanNENs vascularization and disease aggressiveness and prognosis has been reported [5,6].

Based on the presence or absence of clinical syndromes associated with hormonal hypersecretion, PanNENs are classified into "functioning" (F) and "non-functioning" (NF), the latter accounting for the vast majority of PanNENs (60-90%) [7]. Due to their specific clinical presentations and related syndromes, F-PanNENs are frequently associated with an early disease detection. On the other hand, patients with NF-PanNENs usually present no or aspecific symptoms (e.g., abdominal distension, abdominal pain) in the initial stages, causing a delay in the diagnosis. Indeed, NF-PanNENs are often identified later in the course of the disease, when clinical symptoms associated with mass effect or metastatic involvement, appear. Overall, 12-74% of PanNENs have distant metastases, mainly hepatic, at initial diagnoses, and are associated with poorer prognosis [8,9] .

According to latest WHO classification [10,11], PanNENs are categorized into well-differentiated pancreatic neuroendocrine tumors (PanNET grade 1, PanNET grade 2, PanNET grade 3), poorly differentiated pancreatic neuroendocrine carcinomas (PanNECs small-cells, PanNECs large-cells), and mixed Neuroendocrine–Non-neuroendocrine Neoplasms (MiNENs).

PanNENs are traditionally regarded as rare neoplasms, with an annual incidence of less than 1 case per 100'000 inhabitants, and they account for less than 3% of all pancreatic tumors [12]. However, these data seem to underestimate the real occurrence of PanNENs. Indeed, autoptic studies indicate a prevalence of small PanNENs that ranges between 0.8–10%, suggesting that these lesions remain

frequently undetected [13,14]. Over the last two decades a significant rise in the diagnosis of early-stage PanNENs has been observed [15,16], primarily due to the increase in the incidental discovery of small asymptomatic lesions during cross-sectional imaging performed for disease-unrelated causes.

PanNENs diagnostic workup mainly relies on biochemical analyses, imaging examinations, including radiological imaging, functional imaging and endoscopic ultrasound, and pathology; this latter is essential for the establishment of PanNEN final diagnosis [17,18]. Tumor localization, staging, grading, and characterization are the ultimate goals of the diagnostic process, upon which final clinical decision-making is based on.

The selection of therapeutic strategies (i.e., active surveillance, surgical resection, medical treatment) for PanNENs primarily hinges on the degree of disease extension and aggressiveness [18]. Nevertheless, due to PanNENs biological heterogeneity and to the lack of valid preoperative prognostic factors available, the risk of overtreatment and undertreatment remains substantial [2,19–22]. Indeed, an accurate diagnosis and, consequently, an appropriate therapeutic management of these lesions remain challenging.

In this context, new developments have been recently emerging as possible diagnostic tools for PanNENs, including a deeper understanding of PanNENs biology, advancements in imaging techniques and the use of artificial intelligence. These progresses have improved PanNENs identification, resulting in early disease detection and characterization, and paving the way towards early prediction of tumor aggressiveness and patients' prognostic stratification.

The aim of this review is to present a throughout overview of the diagnostic approaches for PanNENs, focusing on the latest advances. A comprehensive literature search on PubMed and Embase was undertaken using key words "pancreatic neuroendocrine tumors", "diagnosis", "imaging", "pathology", and "biomarkers", from inception to October 2023.

2. Circulating biomarkers

2.1. Biochemical markers

Specific blood/urine biomarkers play a pivotal role in the diagnostic workup of F-PanNENs. Indeed, F-PanNENs are associated with the overproduction of a specific hormone, leading to distinct clinical syndromes and guiding diagnostic testing [23–25]. Key biomarkers for F-PanNENs diagnosis are summarized in **Table 1.**

In presence of clinical suspicion of insulinomas, the most common F-PanNENs, key diagnostic criteria encompass inappropriately elevated plasma insulin, C-peptide, and pro-insulin levels alongside with low blood fasting glucose level. Regarding gastrinomas, which comprise 20–50% of F-PanNENs, assessment of serum gastrin levels is critical. Indeed, a significant increase in fasting serum gastrin levels, in combination with a low gastric fluid pH, are indicative of gastrinomas. In suspicions of ACTHoma or carcinoid tumors, specific urine tests should be conducted for diagnostic confirmation. For ACTHoma, a 24-hour cortisol test is necessary, whereas carcinoid tumors require a 5-hydroxyindoleacetic acid (5-HIAA) test as a standard procedure [24,25]. These biomarkers play a crucial role not only for F-PanNENs initial diagnosis, but also in monitoring treatment response and detecting disease recurrence.

Regarding NF-PanNETs, biochemical diagnosis relies on nonspecific diagnostic biomarkers, including chromogranin-A (CgA), pancreatic polypeptide (PP), and neuron-specific enolase (NSE). In addition, elevated serum levels of carcinoembryonic antigen (CEA), carbohydrate antigen19-9 (CA19–9), CA125, and alpha-fetoprotein (AFP) have been reported to be associated with PanNECs [23,26–29]. CgA represents the sole circulating biomarker recommended by ENETS guidelines for diagnosis and follow-up [18]. CgA has demonstrated an overall good test performance in NETs diagnosis, with a sensitivity and specificity of 73% and 95%, respectively [30]. Moreover, circulating CgA levels have proved to be strongly correlated with disease burden, presence of metastases, and disease recurrence [31–33]. In addition, concomitant assessment of PP and CgA was reported to increase the reliability of the measurements and PanNENs diagnosis [34]. Nonetheless, the accuracy

of CgA and its clinical utility are still controversial [35–39]. Notably, CgA levels may be affected by several conditions unrelated to PanNETs, as the use of proton-pump inhibitors, somatostatin analogues, hypertension, kidney dysfunction and various benign and malignant diseases [37,40,41]. Moreover, tumor differentiation, tumor functional status, and the presence of inherited syndromes (e.g., MEN1) can alter CgA diagnostic performance [39]. Finally, poor laboratory assay reproducibility poses a significant challenge in CgA values interpretation.

Due to the numerous limitations associated with CgA assessment, new biomarkers have been tested. In this context, the clinical utility of vasostatin-1 (VS-1), a fragment derived from the cleavage of CgA, was recently investigated [36,42,43]. Remarkably, unlike CgA, VS-1 levels are not affected by PPIs. Andreasi et al. [42] observed a positive correlation between high VS-1 levels and the presence of features of aggressiveness at final histology, such as nodal metastases and microvascular invasion. In addition, in a cohort of 35 patients who underwent surgery for NF-PanNETs, VS-1 showed a higher accuracy compared to CgA in assessing the effectiveness of surgical resection [36]. Notably, the series [36] reported no significant association between surgical efficacy and the levels of pancreastatin (PST) and vasostatin-2 (VS-2), other CgA-derived fragments.

Angiogenic factors, mainly Angiopoietin-2, have been recently examined as potential biomarkers [3,44–46]. Ang-2, by binding to its receptor, Tie-2, promotes endothelial cell survival and vascular remodeling [47]. Srirajaskanthan et al. [48] demonstrated an elevated Ang-2 serum levels in patients with NENs compared to healthy patients. Consistently, Detjen et al. [46] found similar results. Furthermore, metastatic GEP-NENs showed higher circulating Ang-2 levels with respect to localized lesions [44,46,48]. Despite these promising results, further prospective studies are warranted.

2.2. Liquid biopsies

Liquid biopsies encompass the noninvasive analyses of circulating tumor-derived material, the latter retrieved from peripheral blood or other bodily fluid. In this context, the efficacy of NETest, a multigene liquid biopsy, has been recently assessed.

NETest, or Neuroendocrine Tumor Gene Expression Test, is a specific blood-based molecular diagnostic test that quantifies multiple mRNA gene transcripts associated with neuroendocrine tumors [49]. The NETest is designed to evaluate 51 transcriptomic signatures of NENs in blood samples using polymerase chain reaction (PCR). These transcripts are then analyzed according to a 'NET-omic' signature, and a mathematical algorithm calculate a NETest score that ranges from 0 to 100%, providing an objective stratification of the tumor [37,50]. NETest has demonstrated its value in detecting disease recurrence, assessing surgical efficacy, and monitoring treatment response [32,50–55], with a mean diagnostic accuracy of approximately 95% [50,55]. Additionally, NETest has shown significant superiority in disease detection and in evaluating surgical efficacy when compared to CgA [53,56,57]. Indeed, NETest demonstrated a higher diagnostic accuracy and ability in identifying residual or recurrent disease after surgical resection, compared to CgA serum levels. Furthermore, unlike CgA, NETest is not affected by proton pump inhibitors or diet [55]. Nonetheless, the limitations of this assay include test accessibility, reproducibility, and cost-effectiveness.

In addition to NETest, other multianalyte biomarkers, including micro-RNA (miRNA) and circulating tumor DNA (ctDNA) analyses using PCR and next-generation sequencing (NGS) techniques, have been explored [58–61]. Moreover, the potential prognostic significance of circulating tumor cells (CTCs) has been investigated [32,62]. Despite their innovative concepts and technological approaches, further in-depth analyses of these markers and methodologies are required before they can be considered for clinical applications.

3. Radiological Imaging

Cross-sectional imaging, such as computed tomography (CT) and magnetic resonance imaging (MRI), represents a pivotal tool for PanNENs localization, staging and characterization. Intravenous contrast is essential to enhance the diagnostic efficacy of both CT and MRI [63].

CT represents the first-line imaging technique for PanNENs diagnostic work-up and staging [18,64], thanks to its good sensitivity and specificity in disease detection, wide availability, and short imaging time. PanNENs are generally hypervascular lesions and show enhancement in the arterial phase at contrast enhanced (CE) CT (**Figure 1A)**. Recently, the pattern of enhancement at CE-CT early (arterial) and late (venous) phases has been shown to be strongly associated with disease aggressiveness and patient's prognosis [65–69]. In particular, CE-CT arterial hypo-enhancement and late washout have been recognized of being significantly correlated with low microvessel density and markers of aggressiveness (i.e., grade, nodal metastases) at pathological evaluation in patients undergoing surgery for PanNENs. In addition, CE-CT is the most accurate cross-sectional imaging modality for the assessment of vascular infiltration [70], delineating tumor resectability.

MRI provides comprehensive anatomical information regarding the primary tumor site and extent, and can be employed as a complementary tool to CT examination [64]. Of note, diffusion weighted MRI, thanks to its greater image contrast and functional information, demonstrated a high sensitivity in identifying small PanNENs (e.g., insulinomas) [71,72]. At MRI evaluation, PanNENs typically show intense arterial enhancement, and are hypointense at T1-weighted sequences and hyperintense at T2-weighted sequences. Regarding disease staging, MRI, especially in diffusion weighted sequences, proved to be the most sensitive technique for detection of liver metastases [73–77]. The use of liver-specific agents (e.g., gadoxetate disodium) can also be employed to further enhance MRI detection yield in this context [78–80]. In addition, MRI represents the technique of choice for the assessment of the relationship of the neoplastic lesion to the main pancreatic duct, providing valuable information for surgical planning (i.e., enucleation in presence of insulinomas or small NF-PanNETs). Furthermore, MRI plays a significant role in the surveillance of patients with small, indolent PanNENs as well as with MEN-1 syndrome, by minimizing radiation dose exposure.

CE-CT and MRI images of a patient diagnosed with metastatic PanNET are shown in **Figure 2**.

3.1. CT and MRI Radiomics

Radiomics is a high-throughput data mining process, consisting in the extraction and analysis of quantitative features from medical images using statistical and/or deep learning techniques, with the intent to generate models predicting histological characteristics and clinical outcomes [81–83]. Recent experiences have tested the feasibility of extracting and exploiting radiomic features (RF), defined as image-based biomarkers, in patients affected by PanNENs, reporting promising results. Most studies were conducted investigating CT data, and only a few analyzed MRI images [84–90]. The possible role of MRI-derived radiomics in predicting tumor grade, vascular involvement, and nodal and liver metastases in PanNETs was assessed and confirmed by De Robertis et al. [86]. Regarding CT, a retrospective study conducted by Benedetti et al. [90] demonstrated that multiple CT RFs had significant discriminatory power in identifying PanNETs histopathological characteristics including tumor grade, nodal disease, microscopic metastasis, and vascular invasion. Guo et al. [91] confirmed the ability of CT RFs in predicting PanNET grading, thus facilitating treatment selection in this setting. In addition, significant differences in RFs between grade 1 and grade 2/grade 3 PanNETs were reported by recent studies [92–95]. Furthermore, a retrospective series including 75 patients with NF-PanNET reported that CT-derived radiomic features are superior with respect to EUS-FNA, in accurately identifying G2-G3 lesions (sensitivity, 80.8% *vs* 42.3%) [96]. Lastly, two recent experiences [88,97] demonstrated that preoperative identification of histological features of PanNENs, including grading [97], presence of distant metastases and vascular invasion [88], could be achieved by combining radiomics with clinical characteristics. Furthermore, McGovern et al. [98] investigated the relationship between PanNETs CT-derived radiomics and genetic, an emerging discipline referred as radiogenomics [99], proving the ability of RF in predicting the alternative lengthening of telomeres (ALT)-positive phenotype, providing addition prognostic information.

4. Functional Imaging

Functional imaging, including ^{68}Gallium (Ga)-DOTA-peptide PET/CT and Fluorine-18 fluorodeoxyglucose ([^{18}F]-FDG) PET/CT, plays a crucial role in accurately assessing disease extent, both during the initial staging and follow- up, as well as in identifying occult primary lesions [100]. In

PanNENs, the execution of a whole-body somatostatin receptor (SSTR) imaging, with ^{68}Ga PET, is recommended during the diagnostic work-up, preoperative assessment and restaging, representing the standard functional modality to evaluate well differentiated PanNENs [17,64,101]. Indeed, PanNENs typically express somatostatin receptors, allowing for excellent detection and characterization of these lesions using somatostatin analogs (SSA) coupled with radionuclide tracers (**Figure 1B**). [^{68}Ga]Ga-DOTA-peptide PET images are fused with CT to further improve their anatomic specificity and the possible use of [^{68}Ga]Ga-DOTA-peptide PET/MRI has also been recently investigated [102–104]. [^{68}Ga]Ga-DOTA-peptide PET/CT demonstrated a high sensitivity in identifying PanNET disease [105], representing the most accurate imaging modality for detection of distant metastases [63]. Recently, new radiotracers, including ^{64}Cu, have been proposed as novel functional imaging technique, with promising results [106–108]. Besides aiding in disease localization and staging, [^{68}Ga]Ga-DOTA-peptide PET provides valuable information for treatment selection [63]. Indeed, the levels of radiotracer uptake correlate with the degree of SSTR expression and the response to peptide receptor radionuclide therapy (PRRT) [109,110]. Moreover, the intensity of radiotracer uptake, defined as Standardized Uptake Values (SUVmax), provides relevant prognostic information, being correlated with clinical and pathological features [111,112]. False positive [^{68}Ga]Ga-DOTA-peptide PET uptakes may also occur, and differential diagnosis with other conditions associated with SSTRs overexpression should be considered (i.e., pancreatic uncinate process activity, splenosis, solid serous cystadenoma, and metastases from renal cancer) [113]. On the other hand, false negative results may be encountered in the presence of small insulinomas, which are characterized by a reduced SSTR expression. In this setting, PET/CT with radiolabeled glucagon-like peptide-1 receptor (GLP-1R) analogue, named exendin-4, proved to accurately detect benign insulinomas, as these lesions frequently overexpress this receptor [114,115]. Moreover, ^{68}Ga PET examination may not yield informative results when dealing with high-grade (e.g., ki67>10%), poorly differentiated PanNECs due to their decreased SSTR expression. Conversely, these lesions tend to exhibit increased metabolic activity, making ^{18}F-FDG a better tracer to be used for their identification [116,117] (**Figure 3**). The use of dual tracer PET/CT with [^{68}Ga]Ga-DOTA-peptide and [^{18}F]-FDG is advantageous in evaluating metabolic phenotype and discriminating between grade 3 PanNETs, which exhibit higher [^{68}Ga]Ga-

DOTATE uptake, and grade 3 PanNECs, which display higher [^{18}F]-FDG uptake [63,118,119]. However, the precise role of the dual-tracer approach in the diagnostic work-up of PanNENs remains unclear [120]. Notably, [^{18}F]FDG uptake appears to be associated with poorer prognosis, independently of tumor grade [118,119,121–123]. Nevertheless, the routine use of this imaging modality is not recommended. Its use should be contingent upon a case-by-case basis, mainly for advanced PanNENs, and reserved for situations where a change in therapeutic decision is expected upon its findings [17]. Finally, ongoing investigations are exploring new tracers (e.g., [^{68}Ga]Pentixafor, [^{68}Ga]Ga-NODAGA-JR11, [^{18}F]AlF-OC) in an effort to enhance the prognostic potential of functional imaging; however, no consistent results are currently available [118,124].

4.1. [^{68}Ga]Ga-DOTA-peptide and [^{18}F]FDG PET Radiomics

Radiomics has been recently introduced in the context of PanNENs functional imaging [125,126]. Preliminary exploratory studies have examined the ability of radiomic features extracted from [^{68}Ga]Ga-DOTA-peptide and [^{18}F]FDG PET/CT images to predict tumor aggressiveness and treatment response [127–131]. Mapelli et al. [127] demonstrated the significance and utility of texture features derived from [^{68}Ga]Ga-DOTATOC and [^{18}F]FDG PET/CT images in predicting tumor stage, angioinvasion, and nodal metastases. Ma et al. [132] showed the capacity of [^{68}Ga]Ga-DOTATATE PET/CT – derived features to accurately discriminate grade 1 from grade 2/ grade 3 PanNETs, contributing to clinical diagnosis and patients' treatment management. Notably, Mapelli et al. [133] also reported the ability of [^{68}Ga]Ga-DOTATOC PET extracted parameters in predicting DAXX/ATRX loss of expression in patients with PanNETs. Additionally, two recent experiences [130,132], indicated a strong correlation between [^{68}Ga]Ga-DOTATOC PET/MRI-derived parameters and tumor aggressiveness [134] and nodal involvement [130]. Furthermore, recent research has explored the potential of [^{68}Ga]Ga-DOTATOC PET/CT radiomic features for predicting treatment response to PRRT in this context, reporting promising results [128,135] .

5. Endoscopic ultrasound (EUS)

Endoscopic Ultrasound (EUS) represents the most accurate imaging technique for diagnosing small PanNENs [77,136], with a sensitivity of 93% and specificity of 95%[137]. EUS allows for precise tumors localization and extent. On EUS, PanNETs typically appear as well-defined, round, hypoechoic, homogenous and well-vascularized lesions. EUS can also assess for potential resectability by evaluating local staging, disease multifocality, relationship to the pancreatic duct and lymphovascular involvement [138,139]. In this setting, EUS was recently reported to be the preoperative diagnostic imaging with the highest specificity (98%) in identifying local nodal metastases in NF-PanNETs with respect to CE- CT, and [^{68}Ga]Ga-DOTATOC PET [139]. One of the advantages of EUS is the possibility to use different tools to increase the diagnostic accuracy (e.g., elastography) and to perform operative procedures.

5.1. EUS-guided FNA/FNB

Other than describing morphologically the lesions, EUS offers the possibility to achieve a pathological diagnosis and grading [11,140,141], and it should be performed whenever possible [18,142].

Indeed, tissue sampling can be obtained by EUS-guided fine-needle aspiration (FNA) and fine-needle biopsy (FNB) [138]. The difference between FNA and FNB is related to the shape of the distal part of the needle that allows to obtain, respectively, a cytological or a micro-histological specimen. Given the crucial role of preoperative grading in PanNENs therapeutic selection, several reports have investigated the correspondence of EUS-FNA and EUS-FNB grading with final histology on surgical specimen. Regarding EUS-FNA, an accuracy of 89% and of 70-75% were observed for PanNENs diagnosis and grading, respectively [143–145]. This could be due to the partial sampling of the lesion by EUS-FNA, and so the neoplastic sample may not be representative of the whole tumor because of intratumoral heterogeneity. In addition, EUS-FNA sampling adequacy could be affected by other factors as tumor location and presence of stromal fibrosis [146]. On the other hand, EUS-FNB is more

likely than FNA to provide adequate samples for grading, with a diagnostic adequacy of 98.3% and a Ki67 assessment adequacy of 84.7% [138]. Moreover, a recent meta-analysis found that the grading concordance between preoperative EUS samples and surgical specimens is better with EUS-FNB (84.2%) compared to EUS-FNA (79.5%) [147]. The higher accuracy of EUS-FNB, especially in presence of lesions <2cm, was also confirmed by a recent study by Crinò et al. [148].

Recently, in the attempt to override tissue sampling limitations, the possible role of next generation sequencing (NGS) analysis on EUS-FNA and EUS-FNB samples has been tested. Both the procedures have demonstrated to be able to provide sufficient tissue for NGS to identify somatic mutations that influence tumor behavior (i.e., MEN1, DAXX, ATRX, and ALT) [149–152]. However, also in this setting, EUS-FNB have shown to have a significantly higher-quality DNA and RNA yield with respect to EUS-FNA, [153] with a DAXX, ATRX, and ALT status concordance between EUS-FNB and primary tumor specimen of 95.1%, 92.7%, and 100%, respectively. Furthermore, EUS-FNB mutational detection was found to be associated with tumor aggressiveness and disease prognosis [150].

Finally, a single experience [154] has assessed the safety and efficacy of Confocal laser endomicroscopy (CLSM) in the diagnosis of PanNETs. CLSM is a novel endoscopic method that uses fluorescent dyes to enable a real-time in vivo histopathological evaluation of the lesion, without tissue sampling. Despite the promising reported results, CLSM is an operator-dependent technique that can be performed only in tertiary referral centers, and objective definition for intra- and inter-observer agreement, as well as procedure methodology, still need to be tested.

5.2. EUS elastography (EUS-E)

EUS-E is a novel technique allowing real-time evaluation of tissue stiffness. Elastographic images reflect tissue elasticity, which has been proved to correlate with the histopathological features of the lesion [155]. In the clinical setting, strain and shear wave elastography are mainly used for pancreatic diseases; however, only the former one has been tested on PanNENs [143,156]. The evaluation

methods of strain elastography are classified into color pattern diagnosis, strain ratio (SR), and histogram analysis [143]. Promising results have been reported on the accuracy of E-EUS for the differential diagnosis of solid pancreatic tumors [155–158]. Indeed, on EUS-E, a significantly higher strain ratio was observed in malignant lesions (i.e., pancreatic adenocarcinoma) when compared to benign lesion as small PanNETs [159] . Iglesias García et al. [157] reported a sensitivity of 100% and specificity of 88% of quantitative elastography (SR) in differentiating pancreatic adenocarcinoma from PanNETs. Quantitative elastography is able to grossly discriminate PanNENs aggressiveness, but sensitivity and specificity are still low (67% and 71%, respectively) [160]. Furthermore, studies fully dedicated to the role of EUS-E in PanNENs are still lacking and further technique refinement is necessary [156].

5.3. Contrast-enhanced harmonic EUS (CE-EUS)

CE-EUS is another EUS ancillary technique that allows real-time evaluation of tumor microvascularization. CE-EUS has shown a high sensitivity and specificity in PanNENs identification [161]. Typically, PanNENs are characterized by an early hyperenhancing pattern with a rapid washout, reflecting their increased arteriolar vascularity. In addition, CE-EUS have demonstrated the ability to predict PanNENs aggressiveness [67,162,163], with a sensitivity of 96% and a specificity of 82% [162]. Indeed, hyperenhancing CE-EUS pattern is typical of well-differentiated low grade PanNENs, while an hypoenhancing pattern with late washout reflects a low microvessels density, that is associated with the presence of features of aggressiveness (**Figure 4**). Recently, CE-EUS with quantitative perfusion analysis [164] have also demonstrated to be a valid and objective tool for PanNEN diagnosis and grading [165,166]. Moreover, Saftoiu et al. [167] have reported encouraging preliminary results on the use of artificial intelligence (AI) for EUS diagnosis of pancreatic tumors. Nonetheless, further studies are warranted.

6. Pathology

Pathological examination is essential for a definitive diagnosis of PanNENs, and it can be performed on either surgical specimen, histological biopsy, or fine needle aspirate. A comprehensive histological assessment not only confirms the neuroendocrine nature of the tumor but also determines its grade and degree of differentiation. In particular, histo-morphological growth pattern and cytology enable PanNENs identification. PanNENs neuroendocrine phenotype is proven by immunohistochemical staining for the neuroendocrine markers synaptophysin and/or chromogranin A (CgA), as well as insulinoma-associated protein 1 (INSM1) [17,168,169]. Based on the latest WHO classification [10,11,169], PanNENs are divided into well differentiated pancreatic neuroendocrine tumors (PanNETs G1-G2-G3) and poorly differentiated pancreatic neuroendocrine carcinomas (PanNECs G3), based on tumor grading and cell morphology (**Table 2**). Pathological distinction between PanNETs G3 and PanNECs is pivotal for an appropriate therapeutic management. Indeed, recent studies [170] recognize PanNECs as separate entities from PanNETs G3, characterized by different cells of origins, molecular and biological underlying mechanisms, as well as genetic profiles.

Tumor grading is dependent on mitotic activity (number of mitoses per 2 mm^2) and Ki67 proliferative index. When Ki67 and mitotic count are discordant, the parameter classifying the tumor in the higher-grade category is considered. Neoplasms characterized by the coexistence of a neuroendocrine and non-neuroendocrine component are defined as mixed neuroendocrine–non-neuroendocrine neoplasms (MiNENs). Additional staining for somatostatin receptors (SSTR), peptide hormones, amines, and the use of molecular markers are considered optional and may be applied based on specific clinical requirements [17,171]. Notably, SSTR staining is indicated in the absence of in-vivo somatostatin imaging studies.

In cases where a clear morphological distinction between well-differentiated (PanNETs) and poorly differentiated (PanNECs) morphology is uncertain, immunohistochemical stains for p53, Rb1, DAXX (death domain-associated protein)/ ATRX (alpha-thalassemia/ mental retardation syndrome X-linked), and MEN1 can be conducted. Indeed, genetic alterations involving TP53 and/or Rb1 are commonly observed in poorly differentiated PanNECs [172], whereas mutations in DAXX/ATRX or MEN1

characterizes PanNETs and identifies well-differentiated lesions with more aggressive behavior [173] (**Figure 5**). MEN1 encodes menin, a protein implicated in chromatin remodeling and gene transcription, and its somatic inactivation represent the most common genetic alteration in PanNETs [174,175]. Mutations in DAXX and ATRX, genes involved in telomerase length maintenance, occur in approximately 40% of PanNETs [176]. Loss of DAXX/ATRX and alternative lengthening of telomeres (ALT) recently proved to be strongly associated with tumor aggressiveness and survival outcomes, highlighting the diagnostic and prognostic utility of their assessment [177–179]. Finally, aberrant activation of the oncogenic mTOR (mammalian target of rapamycin) pathway has been reported in 12-25% of sporadic PanNETs. Analysis of mTOR pathway genes, including PTEN, TSC2, DEPDC5, represents a valuable tool for stratifying patients for treatment with targeted therapies, such as mTOR inhibitors [175,176]. In addition, genetic alterations play a key role in the pathogenesis and diagnosis of familial PanNETs. Although the vast majority of PanNETs are sporadic, 10% are due to inherited syndromes which include multiple endocrine neoplasia type 1 (MEN 1), von Hippel-Lindau (VHL) syndrome, neurofibromatosis type 1 (NF1), and tuberous sclerosis complex (TSC) [7]. Notably, MEN1 germline mutations leads to MEN 1 syndrome, the most frequent familial cause of PanNETs. VHL, NF1 and TSC syndromes are caused by germline pathogenic variants in the VHL, NF1, and TSC1/2 genes, respectively [7,180].

Recently, DNA methylation profile and miRNA are emerging as additional critical factors influencing PanNENs behavior [181–185]. Despite the significant progresses achieved in the understanding of genetic and epigenetic alterations associated with PanNENs [185–187], no routine testing for specific molecular markers is currently recommended [171].

Information regarding disease extent [188], nodal status, presence of lymphovascular and/or perineural invasion, and the status of resection margins should be consistently included in the surgical specimen examination. These features collectively offer valuable prognostic insights and play a crucial role in defining treatment management [7,188–191].

7. Conclusion

The heterogeneous clinical and biological behavior of PanNENs poses a significant challenge in their diagnosis. Nonetheless, a substantial rise in their detection has been reported over the past decade. A deeper understanding of PanNENs biology and advancements in imaging and molecular techniques have played a crucial role in this progress, enabling earlier and more accurate disease detection while offering valuable prognostic information that guides therapeutic decision-making. Several modalities and tests are currently available for a comprehensive PanNENs diagnostic work-up, ranging from newly emerging liquid biopsies and circulating biomarkers to CT/MRI and PET radiomic analyses, as well as the latest advances in EUS-associated procedures. In light of the strengths and the limitations of each technique, a multimodal diagnostic approach is recommended for an appropriate identification, characterization, and management of PanNENs.

8. Expert Opinion

PanNENs are highly heterogeneous neoplasms, from both a biological and clinical standpoint, making their diagnosis and management particularly challenging [2]. Upon clinical suspicion, PanNENs diagnosis mainly relies on three pillars: biochemical markers, pathology, imaging. The latter encompassing radiological imaging (CT/MRI), functional imaging (PET imaging employing ^{18}F-FDG and ^{68}Ga-SSA tracers) and endoscopic US.

Pathological examination plays a definitive role in confirming PanNENs diagnosis and assessing disease aggressiveness [18]. Regarding biomarkers, in the context of NF-PanNETs, the sole recommended non-specific biomarker is CgA, although its diagnostic and prognostic significance represents a matter of debate [18], questioning the routine use of this biombarker in the clinical practice. Imaging techniques constitute the primary modalities for detecting and staging PanNENs, significantly impacting therapeutic decision-making. Concerning radiological imaging, CE-CT serves as the initial imaging modality for primary lesions identification and assessing disease extent [192]. On the other hand, when there is suspicion of liver metastases, MRI, especially with diffusion-weighted sequences, is highly recommended due to its higher sensitivity in detecting such lesions [68,74]. Furthermore, the use of [^{68}Ga]Ga-DOTA-peptide PET and [^{18}F]FDG PET scans is

recommended for a comprehensive PanNEN assessment. [^{68}Ga]Ga-DOTA-peptide PET is considered the standard functional modality for evaluating well-differentiated PanNENs. Poorly differentiated PanNECs benefit more from ^{18}F-FDG PET evaluation, which helps assess disease metabolic activity [18,100,116,192]. Additionally, EUS has demonstrated remarkable sensitivity in localizing small NF-PanNETs and detecting nodal infiltration. Moreover, EUS-FNA and EUS-FNB techniques enable the performance of preoperative pathological diagnosis and grading, further enhancing the diagnostic and prognostic capabilities of this modality in the management of PanNENs [138,140,141]. Therefore, considering both the advantages and constraints associated with each diagnostic modality, a multimodal diagnostic approach should always be preferred, tailored on patients' bases.

In recent years, the understanding of the biology of PanNENs has significantly grown and the diagnostic yield of each of the above-mentioned modalities has remarkably improved, resulting in an increase of PanNENs detection [15,16]. Specifically, an earlier disease identification and a more accurate PanNENs characterization and prognostic stratification have been obtained. In this context, the deeper understanding of the molecular biology of PanNENs, in combination with the recent improvements in the molecular and cytological analyses (i.e., PCR, NGS), has allowed for more precise disease classification [10,11], as well as the identification of new prognostic signatures (i.e., DAXX/ATRX loss, ALT) [177,178], and the introduction of new biomarkers such as NETest [50,52,53,56,57]. Additionally, progress in the field of medical imaging has been helpful in improving the diagnosis of PanNENs. Notably, significant strides have been made in imaging techniques, particularly in the early and noninvasive evaluation of disease aggressiveness. Specifically, improvements in EUS technology, such as CE-EUS [67,161–165] and EUS-E [155–159], and the integration of radiomic analyses into the medical domain, incorporated by both radiological and functional images [84,88,90,97,125,127,130,133], have substantially improved the assessment and the prognostic stratification of these lesions. Moreover, the utilization of novel radiotracers in PET scans, such as ^{64}Cu [106,107], and of new receptor targets (i.e., GLP-1R) [115,118], have expanded and refined the detection ability of this imaging modality. However, these novel techniques have specific limitations, that should be recognized. These include reproducibility, intra- and inter- observer

variations, cost-effectiveness, and the poorly standardized procedural methodology. Lastly, further analyses and validation studies are warranted. Once these limitations will be overcome, the inclusion of these innovative tools in the diagnostic work-up for PanNENs will offer valuable insights for patient stratification and treatment selection, ultimately improving the outcomes of patients.

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Tables

Table 1. Overview of functioning pancreatic neuroendocrine tumors

Tumor	Incidence	Secreted hormone	Clinical features	Biochemical diagnosis
insulinoma	40–55%	insulin	Hypoglycemic symptoms, fasting hypoglycemia, rapid correction with glucose (Whipple's triad)	Insulin ≥ 5 mlU/la Glucose 40 mg/dl C-peptide 0.6 ng/ml Proinsulin > 20 pmol/I for >25% of immunoreactive insulin
gastrinoma	20–50%	gastrin	abdominal pain, gastroesophageal reflux, peptic ulcer disease, diarrhea (Zollinger-Ellison syndrome)	Fasting gastrin level >10 times normal range & gastric pH <2
glucagonoma	rare	glucagone	Rash, necrolytic migratory erythema, vein thrombosis, glucose intolerance, diarrhea	Fasting glucagon > 500 pg/ ml
somatostatinoma	rare	somatostatin	Diabetes mellitus, diarrhea, cholelithiasis, weight loss	Somatostatin-fasting serum level >> upper reference limit
VIPoma	rare	vasoactive intestinal peptide (VIP)	Watery diarrhea, hypokalemia, achlorhydria (WDHA syndrome, Verner-Morrison syndrome)	VIP fasting serum level >60 mol/L
ACTHoma	rare	Adreno cortico topic hormone (ACTH)	Obesity, facial plethora, hypokalaemia, diabetes mellitus, muscle weakness, hypertension, moon facies, hirsutism (Cushing syndrome)	24 h urine cortisol, midnight salivary cortisol, cortisol after 1 mg dexamethasone overnight, ACTH
PanNET tumors causing carcinoid syndrome	rare	serotonin	Diarrhoea, flushing, asthma, valvular heart disease (carcinoid syndrome)	Urine 5-HIAA >50 umol/24 h Elevated plasma 5-HIAA or serotonin

5-HIAA, 5-hydroxyindoleacetic acid

Table 2. WHO classification of Pancreatic Neuroendocrine Tumors [11]

Morphology	**Grade**	**Mitotic count (mm^2)**	**Ki-67 index (%)**
Well-differentiated NETs	G1	<2	<3
Well-differentiate NETs	G2	2-20	3-20
Well-differentiated NET1	G3	>20	>20
Poorly differentiate NECs - Small-cell - Large-cell	G3	>20	>20
MiNEN			

NETs, neuroendocrine tumors; NECs, neuroendocrine carcinomas; MiNEN, mixed neuroendocrine–non-neuroendocrine neoplasia

Figure legends

Figure 1. Contrast-enhanced computed tomography (**A**) and [^{68}Ga]Ga-DOTATOC PET/CT (**B**) evaluation of a small pancreatic neuroendocrine tumor. Typical arterial hyperenhancement (**A**) and avid radiotracer uptake (**B**) can be observed. Informed consent was obtained from the patient for the use of images.

Figure 2. Contrast-enhanced CT (arterial phase, **A**) and MRI (T2 phase, **B**; diffusion-weighted, **C**; liver-specific contrast agent, **D**) of a patient with PanNET liver metastases. Informed consent was obtained from the patient for the use of images.

Figure 3. [^{68}Ga]Ga-DOTATOC PET/CT (**A**) and [^{18}F]FDG PET/CT (**B**) of a patient diagnosed with metastatic pancreatic neuroendocrine tumor (PanNET). Both primary tumor (indicated by the red arrow) and liver metastasis showed a high [^{68}Ga]Ga-DOTATOC uptake (**A**). No [^{18}F]FDG uptake was observed (**B**). At histological examination, a G1 (ki67 1%) PanNET was confirmed. Informed consent was obtained from the patient for the use of images.

Figure 4. Contrast-enhanced endoscopic ultrasound showing two different patterns of arterial enhancement, before (**A-C**) and after (**B-D**) contrast medium injection. A hyperenhancing pancreatic neuroendocrine tumor is shown in panel **B**. A hypoenhancing lesion is shown in panel **D**. Informed consent was obtained from patients for the use of images.

Figure 5. Hematoxylin and eosin stains (**A-B**) and immunostains (**C-D-E-F**) of the primary lesion of a patient diagnosed with metastatic pancreatic neuroendocrine tumor (PanNET) (ypT3 N1 M1). Tumor cells positivity for synaptophysin is shown in panel **C**. Proliferation index (Ki67) was below 1% (**D**). ATRX expression was lost in tumor cells whilst it was well maintained in inflammatory and endothelial cells (**E**). DAXX expression (**F**) was preserved both in the tumor and in the surrounding cells. Informed consent was obtained from the patient for the use of images.

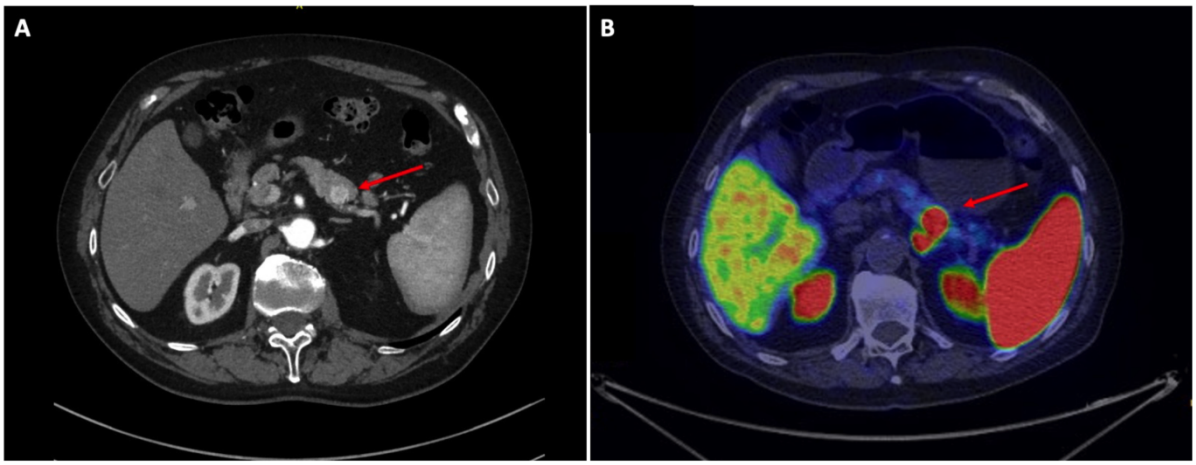

Figure 1

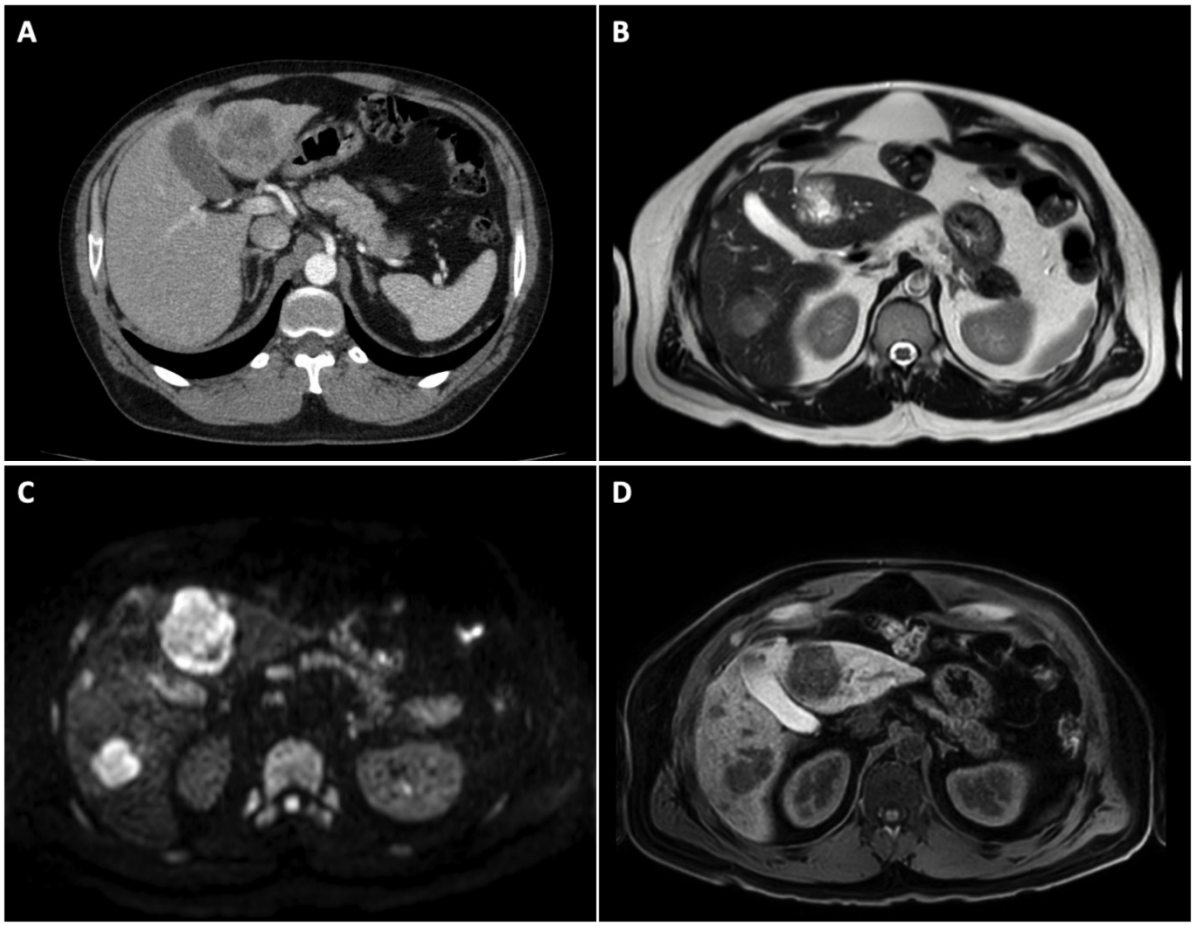

Figure 2

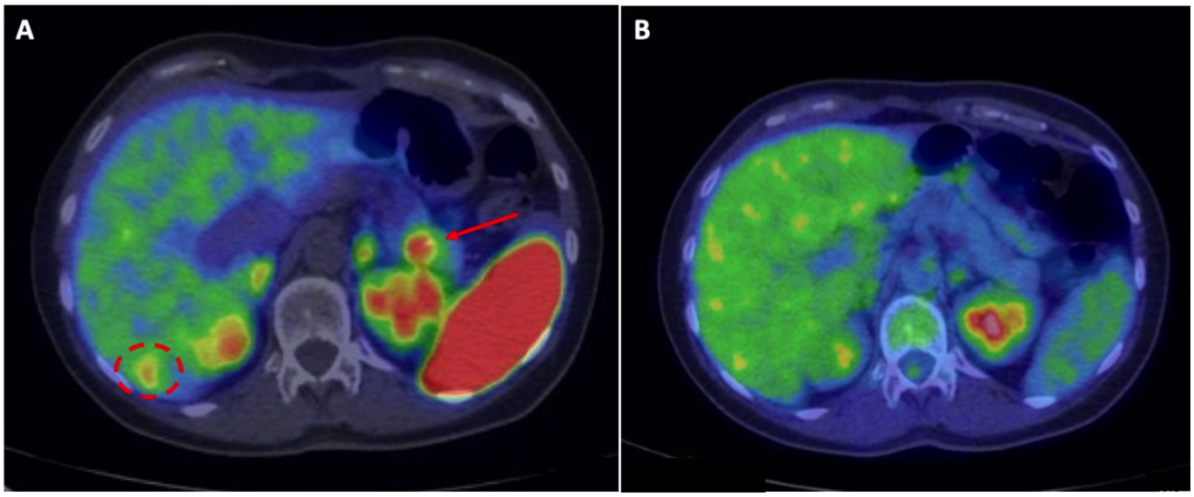

Figure 3

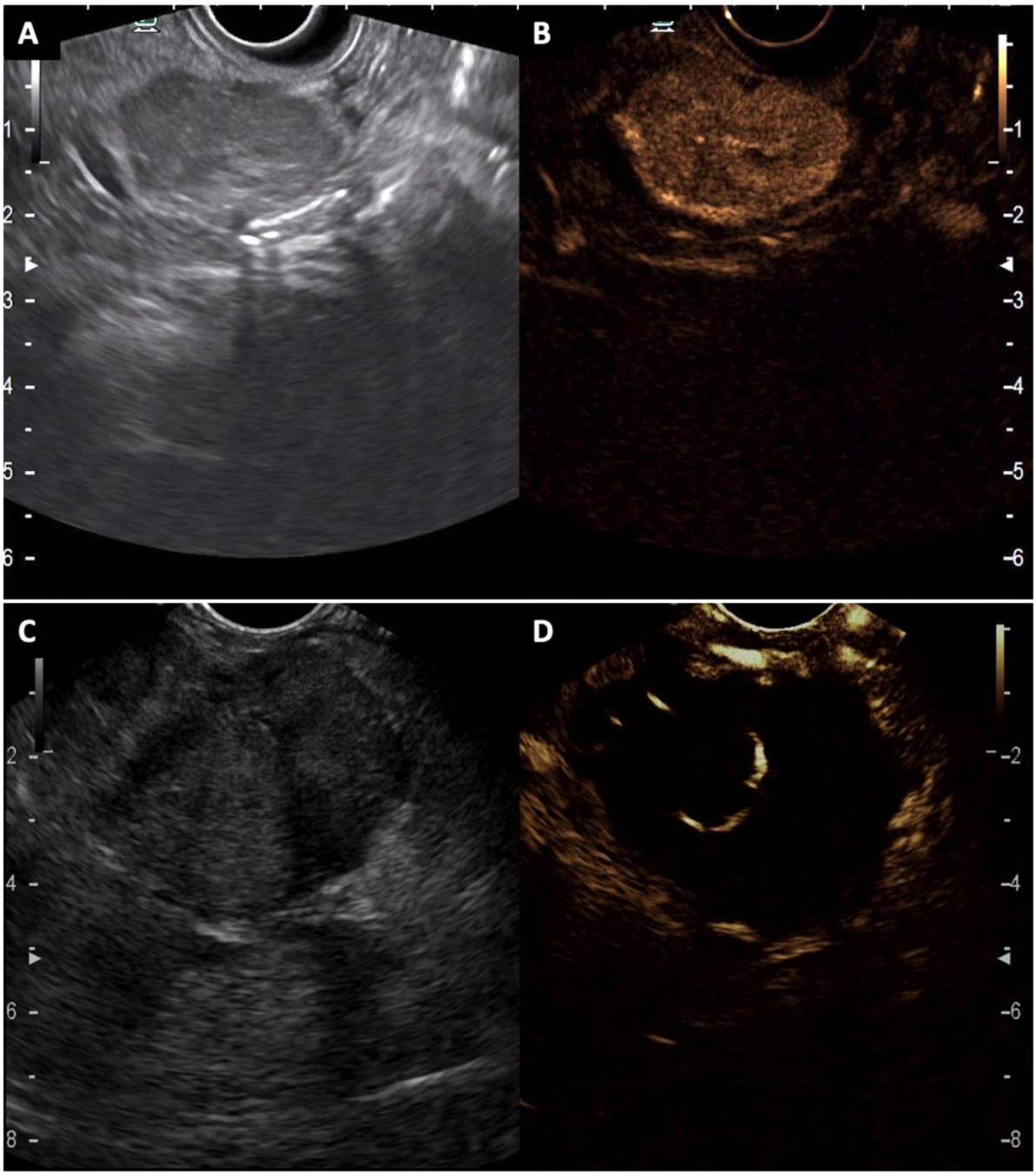

Figure 4

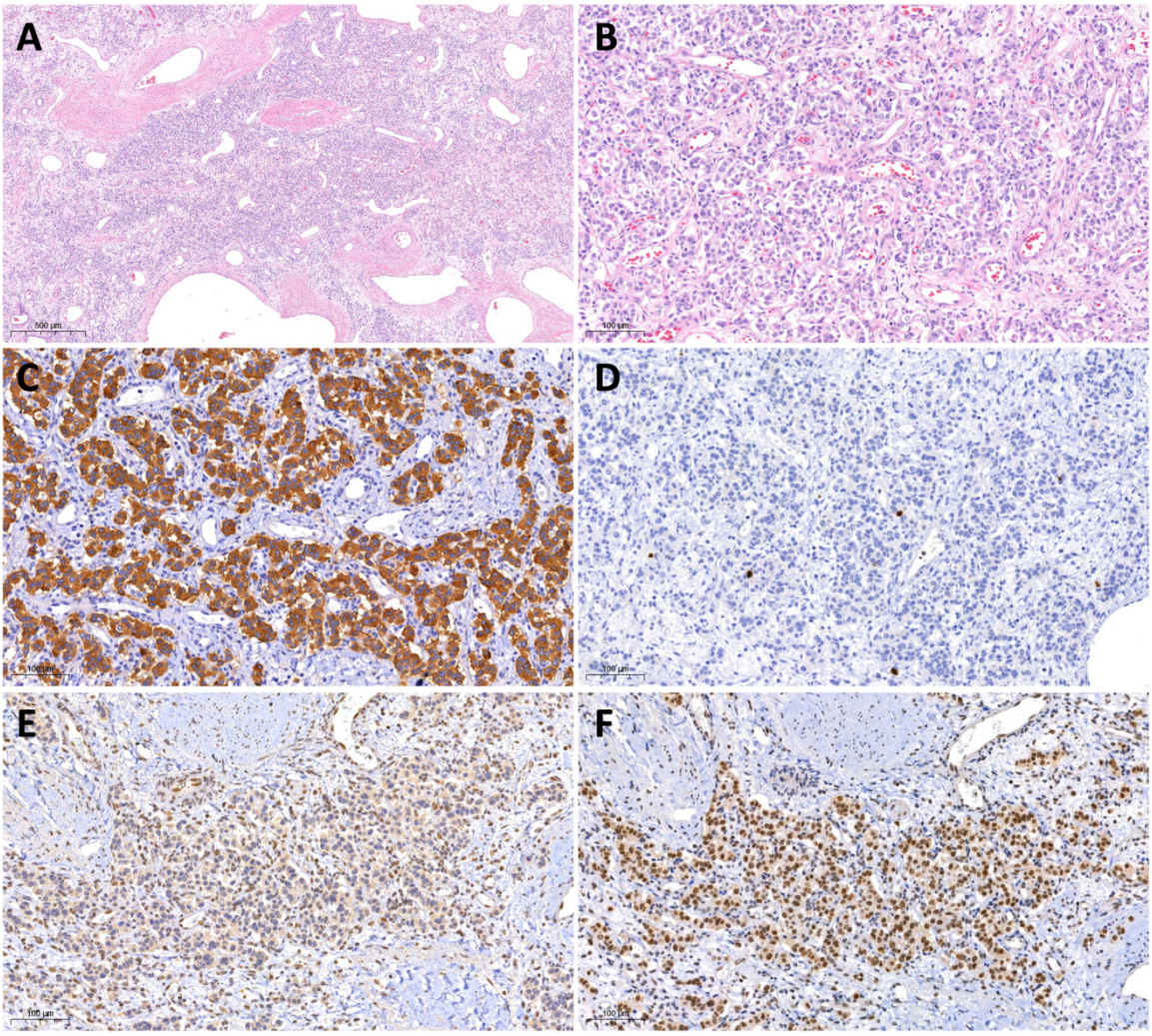

Figure 5