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Neuroendocrine Tumors

Etiology, Diagnosis, and Therapy

Edited by
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MDPI

Neuroendocrine Tumors: Etiology, Diagnosis, and Therapy

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Contents

Editorial

The Increasing Incidence of Neuroendocrine Neoplasms Worldwide: Current Knowledge and Open Issues

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Neuroendocrine neoplasms (NENs) include a heterogeneous group of tumors derived from neuroendocrine cells, most commonly arising from the gastro-entero-pancreatic (GEP) and bronchopulmonary tracts [1,2]. Although NENs have traditionally been considered rare tumors, their incidence has greatly increased over recent decades, partially due to better disease knowledge, the spread of large-scale screening campaigns, and an improvement in diagnostic tools, particularly endoscopy and nuclear medicine. Moreover, the increased incidence was particularly relevant in the stomach (15-fold) and rectum (9-fold), as a reflection of the increased use of endoscopic procedures, including the colorectal screening campaign [3]. Data from the Surveillance, Epidemiology, and End Results Program (SEER) database of the US National Cancer Institute suggested that NEN were more prevalent than hepatobiliary, esophageal, and pancreatic adenocarcinomas combined [4]. According to a recent population-based study that included a total of 43,751 patients, the age-adjusted incidence rate of GEP-NENs increased 6.4-fold from 1975 to 2015; among site groups, the incidence of rectal NENs increased most significantly, whereas as for stage and grade, the incidence increased particularly for the most localized GEP-NENs and G1 neoplasms [5]. Furthermore, reflecting both the rising incidence and the indolent nature of NENs, the 20-year limited-duration prevalence of GEP-NENs increased significantly from 0.00138% in 1996 to 0.03917% in 2015 [5]. Of note, even if the increase in incidence characterized all sites and stages, it was markedly greater for the localized stage, as a consequence of an improved diagnosis of asymptomatic, early stage disease, whilst the proportion of patients with metastatic disease has remained constant over time [6].

Historically, NENs have proven difficult to diagnose, given their nonspecific presentation, which can overlap with other clinical conditions. In this regard, the most typical example is represented by patients with small bowel NENs who often present with vague symptoms and are erroneously diagnosed with inflammatory bowel disease and/or irritable bowel syndrome by general gastroenterologists. As a matter of fact, NENs are often diagnosed when they are already metastatic with a consequent dismal prognosis. However, the improvements in widely used imaging modalities together with endoscopy and nuclear medicine have led to the increased detection of early stage, asymptomatic diseases, which in turn, are characterized by more favorable outcomes [7].

It is, therefore, important to keep in mind that, besides the well-known improvement in diagnostic tools over the years, the increased incidence might be partially dependent on better disease awareness. Furthermore, even if the reportedly improved outcomes with a consequent rise in the prevalence of NENs might be partially attributable to stage migration due to the increase in early stage diagnoses [6], the improvements in systemic therapy have, indeed, also contributed.

Another interesting aspect to be considered as a possible explanation for the registered increase in NENs' incidence is the risk factor exposure; however, only a few factors with

inconclusive results have been identified so far. A family history of any cancer, smoking, and gall-bladder disease/cholecystectomy have been reported to be associated with ~a 1.5-fold increased risk of developing small bowel NENs [8]. Defined risk factors for pancreatic NENs include a family history of multiple endocrine neoplasia type 1 (MEN 1), which confers a 30–80% lifetime risk for developing these neoplasms, smoking, alcohol, and diabetes mellitus [9]. However, solid evidence is lacking regarding the actual role of these risk factors in the development of NENs.

Overall, a significant increase in both the incidence and the prevalence of NENs has been registered over the last decades, particularly in the United States and Canada; however, although to a lesser extent, this positive trend has also been observed in Europe as well as in Asia, thus highlighting the true increase in NENs. The reported differences according to geographic areas might be partially due to heterogeneity of data capture by different registries or to underlying biologic factors, environmental factors, and health care patterns, although a clear-cut explanation is still lacking.

Several improvements have been registered in the diagnostic setting. First, the diagnosis of small bowel NENs, which has always been extremely challenging because of both the lack of specific symptoms at presentation and the poor accessibility of the distal small bowel, has hugely improved with the advent of capsule endoscopy and double-balloon enteroscopy, although solid evidence regarding their actual role in the neuroendocrine setting is still scarce [10]. Ultrasound endoscopy (EUS) represents the diagnostic gold standard for pancreatic NENs and the technique of choice for the loco-regional staging of gastric, duodenal, and rectal NENs [11]. According to the latest European Neuroendocrine Tumor Society (ENETS) Consensus guidelines, EUS was proven to be the most accurate diagnostic technique in pancreatic NEN detection, leading to an up-to-94% sensitivity [12]. Furthermore, advanced EUS techniques may be helpful in the differential diagnosis of pancreatic NENs and the choice of the best-suited treatment. Regarding nuclear medicine, the sensitivity of 68Ga-SSA PET/CT for NEN is >90%, with specificity ranging between 92% and 98% [13]. It plays a pivotal role in the detection of the primary tumor being able to detect even small lesions (i.e., 5 mm) and in the identification of mesenteric lymph nodes and/or local tumor extension to determine the most appropriate surgical approach; it is also necessary for disease staging, being accurate in the detection of distant metastases, particularly bone metastases, in which the presence significantly affects patient's prognosis. Another aspect to be explored is the role of biomarkers in the diagnosis and the follow-up of NENs. Chromogranin A, the most commonly known neuroendocrine general biomarker, is characterized by a low-specificity and might be more useful in the follow-up rather than in the diagnostic setting as a screening tool due to the suboptimal specificity [14–16]. On the other hand, the NETest represents the first successful attempt to provide a multianalyte signature in the blood that has clinical utility in the management of this composite disease. In an ideal world, the societies and guidelines should promote the introduction of novel technology utilizing real-time mathematical analysis of transcriptome-based disease assessment [17].

In terms of therapeutic options, the management of some tumors has changed over the years as there is, indeed, a trend toward less invasive approaches. The most typical example is represented by pancreatic NENs. Considering that, in the most recent decade, we observed a dramatic increase in the diagnosis of small, incidentally discovered, non-functioning pancreatic NENs [3,5], and a clear relationship between the tumor diameter and the risk of malignancy and recurrence has been reported [18], ENET guidelines started to recommend a "wait and see" approach for small asymptomatic non-functioning pancreatic NENs [12], and a European trial is currently ongoing [19]. Furthermore, endoscopic ablative technologies may also be utilized in patients with pancreatic NENs not suitable for surgery or who refused the surgical approach. Duodenal NENs are heterogeneous, still poorly understood tumors as, despite clear differences, their management is treated along with either gastric or, if functioning, pancreatic NENs. Endoscopic resection is increasingly performed instead of surgery. However, duodenal NENs are characterized by a highly

variable prognosis and, despite the small size, can be metastatic in up to 55% of cases, either at diagnosis or thereafter [20]; additionally, considered that conventional imaging has a poor detection rate for loco-regional nodes and micro-metastases in the presurgical setting, the choice of local conservative approaches, including endoscopy or local surgical excision, should be carefully balanced and discussed by a multidisciplinary team and EUS should always be included in the preoperative phase for a more accurate local staging [21]. Rectal NENs have shown a dramatic increase in their incidence, as they are more and more frequently incidentally found during screening colonoscopies. A conservative approach (i.e., endoscopic resection) is recommended for well-differentiated rectal NENs smaller than 10 mm, whereas the best management of tumors between 10 and 20 mm, in which the metastatic risk is intermediate and the endoscopic treatment can be challenging, is still unclear [22]. Several medical therapies for NENs are currently available, including somatostatin analogs, targeted agents, and chemotherapy; a specific mention should be reserved for Peptide Receptor Radiotherapy (PRRT), which based on the recent randomized controlled trial [23], has become an established treatment for malignant metastatic GEP-NENs. As a future perspective, trials focusing on immunotherapy are ongoing for patients with NENs, but no clear-cut data are currently available.

Although NENs are becoming less rare tumors and knowledge of these neoplasms is increasing, given their biological heterogeneity, there is an urgent need for standardized guidelines for the proper management of these neoplasms, which should always be referred to tertiary referral centers. Even if many reports and guidelines regarding NEN management are available and new treatment options for clinical management have been developed, many patients are still referred to specialists with no or low expertise in the neuroendocrine field with a consequent diagnostic delay. It is, in fact, not uncommon that a NEN patient is managed in non-NEN specialized or dedicated structures, where the management of the patient relies on the vision of a single doctor, or on the choice of the patient and/or family to seek second opinions elsewhere. To avoid such scenarios, a multidisciplinary approach and a network between referral centers are necessary to offer every patient the best approach from both a diagnostic and a therapeutic view. It is mandatory to develop novel research strategies to better define diagnostic and therapeutic algorithms, particularly for some specific subgroups of poorly known tumors, including duodenal NENs and functioning tumors. The close cooperation between peripheral and referral centers, and the creation of international disease registries need to be encouraged. The advances in molecular and genetic sciences may be helpful for the application of novel approaches, including neoadjuvant or adjuvant targeted options.

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References

1. Lawrence, B.; Gustafsson, B.I.; Chan, A.; Svejda, B.; Kidd, M.; Modlin, I.M. The epidemiology of gastroenteropancreatic neuroendocrine tumors. *Endocrinol. Metab. Clin. N. Am.* **2011**, *40*, 1–18. [CrossRef] [PubMed]
2. Boyar Cetinkaya, R.; Aagnes, B.; Thiis-Evensen, E.; Tretli, S.; Bergestuen, D.S.; Hansen, S. Trends in Incidence of Neuroendocrine Neoplasms in Norway: A Report of 16,075 Cases from 1993 through 2010. *Neuroendocrinology* **2017**, *104*, 1–10. [CrossRef] [PubMed]
3. Dasari, A.; Shen, C.; Halperin, D.; Zhao, B.; Zhou, S.; Xu, Y.; Shih, T.; Yao, J.C. Trends in the Incidence, Prevalence, and Survival Outcomes in Patients with Neuroendocrine Tumors in the United States. *JAMA Oncol.* **2017**, *3*, 1335–1342. [CrossRef] [PubMed]

4. Yao, J.C.; Hassan, M.; Phan, A.; Dagohoy, C.; Leary, C.; Mares, J.E.; Abdalla, E.K.; Fleming, J.B.; Vauthey, J.; Rashid, A.; et al. One hundred years after "carcinoid": Epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J. Clin. Oncol.* **2008**, *26*, 3063–3072. [CrossRef]

5. Xu, Z.; Wang, L.; Dai, S.; Chen, M.; Li, F.; Sun, J.; Luo, F. Epidemiologic Trends of and Factors Associated with Overall Survival for Patients with Gastroenteropancreatic Neuroendocrine Tumors in the United States. *JAMA Netw. Open* **2021**, *4*, e2124750. [CrossRef]

6. Fraenkel, M.; Kim, M.; Faggiano, A.; de Herder, W.W.; Valk, G.D.; NETwork, K. Incidence of gastroenteropancreatic neuroendocrine tumours: A systematic review of the literature. *Endocr. Relat. Cancer* **2014**, *21*, R153–R163. [CrossRef]

7. Clift, A.K.; Kidd, M.; Bodei, L.; Toumpanakis, C.; Baum, R.P.; Oberg, K.; Modlin, I.M.; Frilling, A. Neuroendocrine Neoplasms of the Small Bowel and Pancreas. *Neuroendocrinology* **2020**, *110*, 444–476. [CrossRef]

8. Rinzivillo, M.; Capurso, G.; Campana, D.; Fazio, N.; Panzuto, F.; Spada, F.; Cicchese, N.; Partelli, S.; Tomassetti, P.; Falconi, M.; et al. Risk and Protective Factors for Small Intestine Neuroendocrine Tumors: A Prospective Case-Control Study. *Neuroendocrinology* **2016**, *103*, 531–537. [CrossRef]

9. de Laat, J.M.; Tham, E.; Pieterman, C.R.; Vriens, M.R.; Dorresteijn, J.A.; Bots, M.L.; Nordenskjöld, M.; van der Luijt, R.B.; Valk, G.D. Predicting the risk of multiple endocrine neoplasia type 1 for patients with commonly occurring endocrine tumors. *Eur. J. Endocrinol.* **2012**, *167*, 181–187. [CrossRef]

10. Rossi, R.E.; Conte, D.; Elli, L.; Branchi, F.; Massironi, S. Endoscopic techniques to detect small-bowel neuroendocrine tumors: A literature review. *United Eur. Gastroenterol. J.* **2017**, *5*, 5–12. [CrossRef]

11. Zilli, A.; Arcidiacono, P.G.; Conte, D.; Massironi, S. Clinical impact of endoscopic ultrasonography on the management of neuroendocrine tumors: Lights and shadows. *Dig. Liver Dis.* **2018**, *50*, 6–14. [CrossRef] [PubMed]

12. Falconi, M.; Eriksson, B.; Kaltsas, G.; Bartsch, D.K.; Capdevila, J.; Caplin, M.; Kos-Kudla, B.; Kwekkeboom, D.; Rindi, G.; Klöppel, G.; et al. ENETS Consensus Guidelines Update for the Management of Patients with Functional Pancreatic Neuroendocrine Tumors and Non-Functional Pancreatic Neuroendocrine Tumors. *Neuroendocrinology* **2016**, *103*, 153–171. [CrossRef] [PubMed]

13. Skoura, E.; Michopoulou, S.; Mohmaduvesh, M.; Panagiotidis, E.; Al Harbi, M.; Toumpanakis, C.; Almukhailed, O.; Kayani, I.; Syed, R.; Navalkissoor, S.; et al. The Impact of 68Ga-DOTATATE PET/CT Imaging on Management of Patients with Neuroendocrine Tumors: Experience from a National Referral Center in the United Kingdom. *J. Nucl. Med.* **2016**, *57*, 34–40. [CrossRef] [PubMed]

14. Rossi, R.E.; Ciafardini, C.; Sciola, V.; Conte, D.; Massironi, S. Chromogranin A in the Follow-up of Gastroenteropancreatic Neuroendocrine Neoplasms: Is It Really Game Over? A Systematic Review and Meta-analysis. *Pancreas* **2018**, *47*, 1249–1255. [CrossRef]

15. Massironi, S.; Rossi, R.E.; Casazza, D.; Conte, D.; Ciafardini, C.; Galeazzi, M.; Peracchi, M. Chromogranin A in diagnosing and monitoring patients with gastroenteropancreatic neuroendocrine neoplasms: A large series from a single institution. *Neuroendocrinology* **2014**, *100*, 240–249. [CrossRef]

16. Massironi, S.; Fraquelli, M.; Paggi, S.; Sangiovanni, A.; Conte, D.; Sciola, V.; Ciafardini, C.; Colombo, M.; Peracchi, M. Chromogranin A levels in chronic liver disease and hepatocellular carcinoma. *Dig. Liver Dis.* **2009**, *41*, 31–35. [CrossRef]

17. Öberg, K. Molecular Genomic Blood Biomarkers for Neuroendocrine Tumors: The Long and Winding Road from Berzelius and Bence Jones to a Neuroendocrine Destination. *Neuroendocrinology* **2021**, *111*, 297–303. [CrossRef]

18. Bettini, R.; Partelli, S.; Boninsegna, L.; Capelli, P.; Crippa, S.; Pederzoli, P.; Scarpa, A.; Falconi, M. Tumor size correlates with malignancy in nonfunctioning pancreatic endocrine tumor. *Surgery* **2011**, *150*, 75–82. [CrossRef]

19. Partelli, S.; Ramage, J.K.; Massironi, S.; Zerbi, A.; Kim, H.B.; Niccoli, P.; Panzuto, F.; Landoni, L.; Tomazic, A.; Ibrahim, T.; et al. Management of Asymptomatic Sporadic Nonfunctioning Pancreatic Neuroendocrine Neoplasms (ASPEN) ≤2 cm: Study Protocol for a Prospective Observational Study. *Front. Med.* **2020**, *7*, 598438. [CrossRef]

20. Massironi, S.; Campana, D.; Partelli, S.; Panzuto, F.; Rossi, R.E.; Faggiano, A.; Brighi, N.; Falconi, M.; Rinzivillo, M.; Delle Fave, G.; et al. Heterogeneity of Duodenal Neuroendocrine Tumors: An Italian Multi-center Experience. *Ann. Surg. Oncol.* **2018**, *25*, 3200–3206. [CrossRef]

21. Rossi, R.E.; Milanetto, A.C.; Andreasi, V.; Campana, D.; Coppa, J.; Nappo, G.; Rinzivillo, M.; Invernizzi, P.; Modica, R.; David, A.; et al. Risk of preoperative understaging of duodenal neuroendocrine neoplasms: A plea for caution in the treatment strategy. *J. Endocrinol. Investig.* **2021**, *44*, 2227–2234. [CrossRef] [PubMed]

22. Ramage, J.; De Herder, W.; Fave, G.D.; Ferolla, P.; Ferone, D.; Ito, T.; Ruszniewski, P.; Sundin, A.; Weber, W.; Zheng-Pei, Z.; et al. ENETS Consensus Guidelines Update for Colorectal Neuroendocrine Neoplasms. *Neuroendocrinology* **2016**, *103*, 139–143. [CrossRef] [PubMed]

23. Strosberg, J.; El-Haddad, G.; Wolin, E.; Hendifar, A.; Yao, J.; Chasen, B.; Mittra, E.; Kunz, P.L.; Kulke, M.H.; Jacene, H.; et al. Phase 3 Trial of 177Lu-Dotatate for midgut neuroendocrine tumors. *N. Engl. J. Med.* **2017**, *376*, 125–135. [CrossRef] [PubMed]

Article

Survival According to Therapy Regimen for Small Intestinal Neuroendocrine Tumors

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Abstract: Introduction: Scarce data exist for therapy regimens other than somatostatin analogues (SSA) and peptide receptor radiotherapy (PRRT) for siNET. We analyzed real world data for differences in survival according to therapy. Patients and methods: Analysis of 145 patients, diagnosed between 1993 and 2018 at a single institution, divided in treatment groups. Group (gr.) 0: no treatment ($n = 10$), gr 1: TACE and/or PRRT ($n = 26$), gr. 2: SSA ($n = 32$), gr. 3: SSA/PRRT ($n = 8$), gr. 4: chemotherapy ($n = 8$), gr. 5: not metastasized (at diagnosis), surgery only ($n = 53$), gr. 6 = metastasized (at diagnosis), surgery only ($n = 10$). Results: 45.5% female, median age 60 years (range, 27–84). A total of 125/145 patients with a resection of the primary tumor. For all patients, 1-year OS (%) was 93.8 (95%-CI: 90–98), 3-year OS = 84.3 (CI: 78–90) and 5-year OS = 77.5 (CI: 70–85). For analysis of survival according to therapy, only stage IV patients (baseline) that received treatment were included. Compared with reference gr. 2 (SSA only), HR for OS was 1.49 ($p = 0.47$) for gr. 1, 0.72 ($p = 0.69$) for gr. 3, 2.34 ($p = 0.19$) for gr. 4. The 5 y OS rate of patients whose primary tumor was resected ($n = 125$) was 73.1%, and without PTR was 33.3% (HR: 4.31; $p = 0.003$). Individual patients are represented in swimmer plots. Conclusions: For stage IV patients in this analysis (limited by low patient numbers in co. 3/4), multimodal treatment did not significantly improve survival over SSA treatment alone. A resection of primary tumor significantly improves survival.

Keywords: neuroendocrine; diagnosis; delay; metastases

1. Introduction

Neuroendocrine tumors of the small intestine (siNET) are a subgroup of gastroenteropancreatic neuroendocrine tumors (GEP NET), a rare entity with an incidence of 2.5/100,000 per year in Europe [1,2]. Among the GEP NET, siNET comprise about one third of cases, rendering them the largest subgroup together with the pancreatic NET (pNET) [1].

siNET are separated in different groups according to their grading, which is mainly deducted from a positive staining for the proliferation marker Ki67 and, to a lesser extent, the mitotic count [3]. Well-differentiated tumors with a Ki67 index of <3% are classified as G1 and, with a Ki67 of 3–20%, as G2. Poorly differentiated neuroendocrine neoplasms (NEN) with a Ki67 index above 20% are, based on morphological features, divided into NET G3 and small- or large-cell neuroendocrine carcinomas (NEC) with implications for treatment and prognosis [3].

In general, treatment options for small intestinal NEN depend on their grading, presence and localization of metastases and somatostatin receptor expression as assessed

by functional imaging, such as somatostatin receptor scintigraphy or somatostatin receptor-directed positron emission tomography–computed tomography (PET-CT) with specific tracers such as ^{68}Ga-DOTATOC or ^{68}Ga-DOTATATE [4–6]. Some treatment modalities for NETs have been investigated in large phase II and III clinical trials; these include surgery for localized and metastatic disease, systemic treatment with somatostatin receptor analogues, or tyrosine kinase inhibitors in metastatic patients, as well as peptide receptor radionuclide therapy (PRRT) in patients with a positive somatostatin receptor expression on the tumor cells [7–13]. In individualized treatment protocols for selected patients or for high-grade tumors (G3), systemic chemotherapy is also an option [14–17]. Locoregional treatment such as transarterial chemoembolization (TACE) can be used in selected patients with predominantly liver metastases or treatment refractory carcinoid syndrome [18,19].

However, since patients with siNET often have a favorable prognosis with 5- and 10-year OS of 67 and 37%, respectively, as a recent meta-analysis reported [20], and undergo several lines of different treatments, the significance of the aforementioned results from clinical trials for real-world situations is sometime difficult to translate, especially in patients beyond classical first- or second-line treatments. In addition, some patients develop metastases with growth patterns that differ from the initial histological result with the need for individualized treatment plans.

The aim of our study was, thus, to assess survival according to treatment in patients with siNET, with an emphasis on metastasized patients and with regard to imaging and laboratory modalities in a real-life setting in a tertiary referral center.

2. Patients and Methods

2.1. Study Design

The present retrospective, single center study was performed to investigate the survival according to therapy in patients with siNET. The study was approved by the institutional review board (internal reference number 319/16) of the University Hospital Frankfurt. Informed consent to participate in the tumor documentation registry was obtained from all living patients. Inclusion criteria of the study were diagnosis with siNET and age $\geq$ 18 years.

2.2. Patient Data

The study database was based on the local electronic hospital charts and was transferred to the local tumor documentation system (Giessener Tumordokumentationssystem, GTDS). A specific NET data set was designed and used for documentation of all patients. The data set included epidemiological and clinical data from 1993 and is explained in detail in Supplementary Table S1. The database has been used for additional analyses without thematically overlapping [21] Data closure and end of follow-up was 15 March 2019).

2.3. Swimmer Plot

The data transformation, data cleansing and plotting algorithm was implemented as interactive Python Notebook (ipynb) in JupyterLab 3.0, using the python 3.7 language with matplotlib 3.2.2, pandas 1.2.0 and numpy 1.19.5. Data operations and the plotting algorithm can be found in the Swimmer_plot.ipnb in the Supplementary Materials.

2.4. Statistical Analyses

Statistical analyses were performed according to international standards and have been described by us and others previously. Analysis was carried out using International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) for Windows (version 22.0; IBM, Chicago, IL, USA), BiAS (version 11, Frankfurt, Germany), and R (version 3.5.1, Vienna, Austria). Categorical variables were described in frequencies and percentages. Continuous variables were represented as a median and range. Continuous variables were compared using the Wilcoxon–Mann–Whitney-U test. All tests were two-sided and p-values $\leq$ 0.05 were considered statistically significant.

3. Results

3.1. Demographics

A total of 145 patients were included in the analysis (Figure 1). A total of 45.5% of all patients were female; the median age was 60 years (range, 27–84). The primary tumor was located in 17.9% in the duodenum; in 6.9% in the jejunum; in 63.4% in the ileum; in 0.7% in Meckel's diverticulum; and in 11.0%, the primary location was NOS (not otherwise specified) in the small intestine.
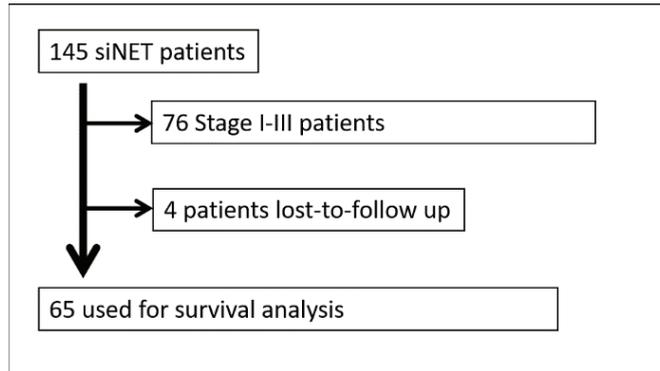
Figure 1. CONSORT diagram.

3.2. Survival According to Therapy

First, we sought to analyze the survival of all patients with siNET. One-, three- and five-year overall survival probability of all patients regardless of their treatment was 93.7%, 84.3% and 77.5%, respectively. The median overall survival (mOS) was 17.13 years (95%-CI: 8.99-NA years), and the median progression-free survival (mPFS) of all patients was 6.21 years (95%-CI: 3.95–8.46 years) (Figure 2).
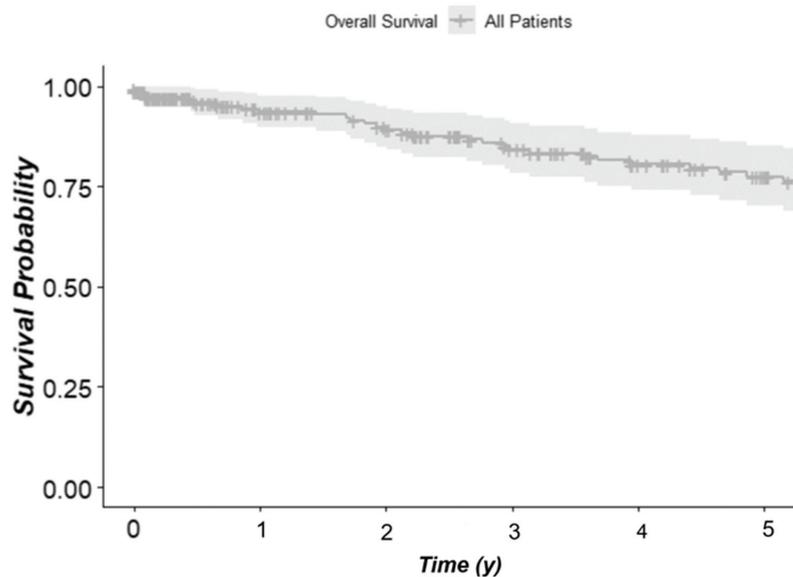
Figure 2. Survival probability, Kaplan–Meier analysis; median overall survival (mOS) was 17.13 years (95%-CI: 8.99–NA years).

However, since NET patients often undergo several treatment lines over many years, which might be overlapping (e.g., somatostatin analogs and locoregional treatment), paused for a certain time or repeated after a few years, which might influence their survival, patients were divided into cohorts depending on the different regimens. Patients in group 5 were not metastasized at diagnosis, and all other patients had metastases.

Group 0: no treatment (*n* = 8), group 1: SSA parallel with TACE and/or PRRT (*n* = 26); group 2: SSA (*n* = 32); group 3: SSA followed by PRRT (*n* = 8); group 4: chemotherapy (*n* = 8); group 5: not metastasized (at diagnosis), surgery only (*n* = 53); group 6 = metastasized (at diagnosis), surgery only (*n* = 10). Each patient's treatment sequence is visualized in Figure 3a–f by a "swimmer plot". Group 0 was excluded from further analyses due to insufficient follow up data (e.g., lost to follow-up, only imaging in our clinic without further contact, only second opinion). The median time of follow up for metastasized patients (*n* = 65) was 2584 days (IQR: 1324; 3634).
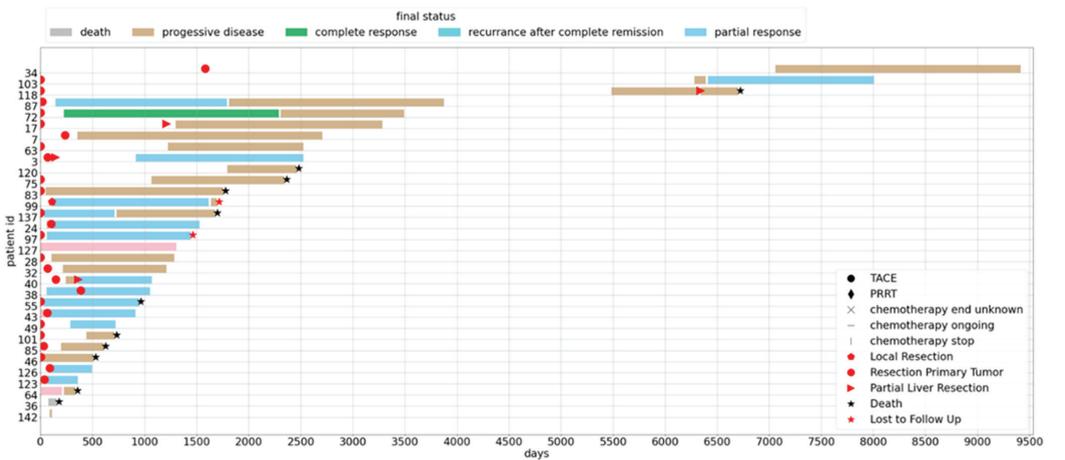
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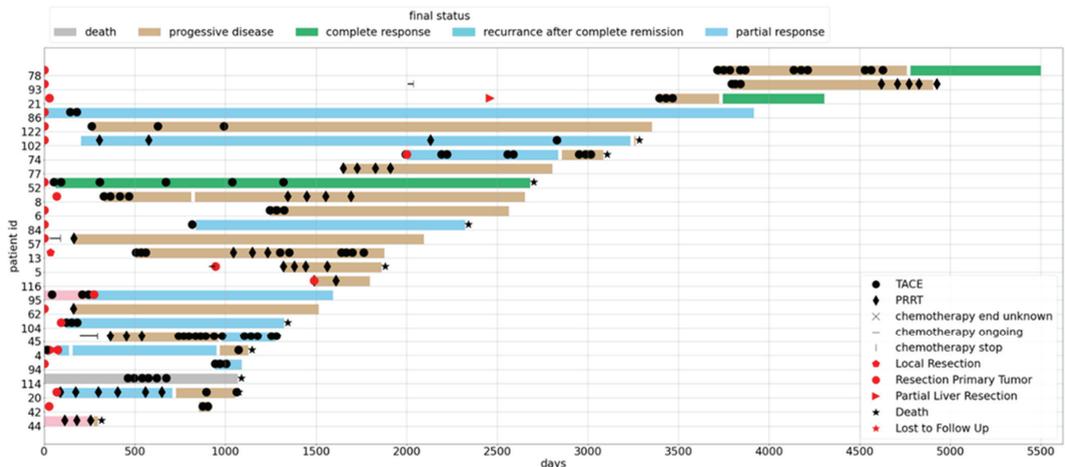
(**b**)

Figure 3. *Cont.*
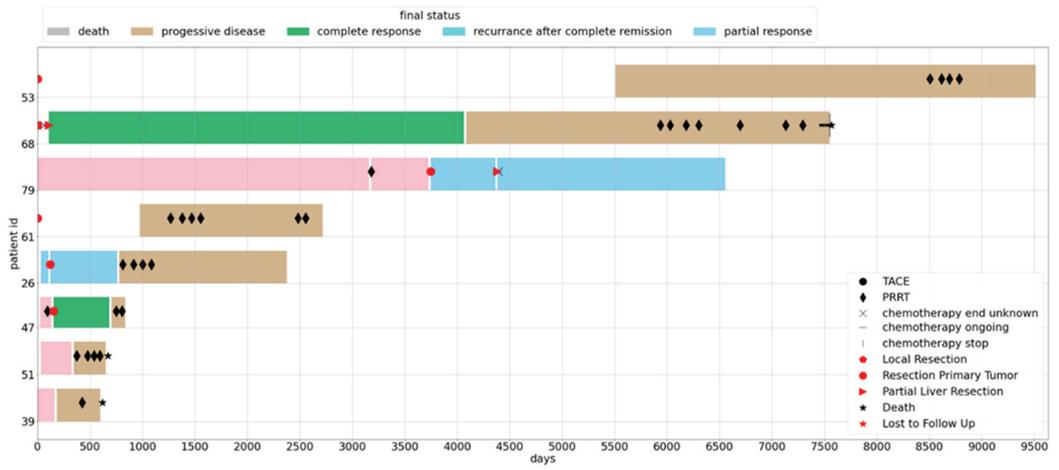
(**c**)
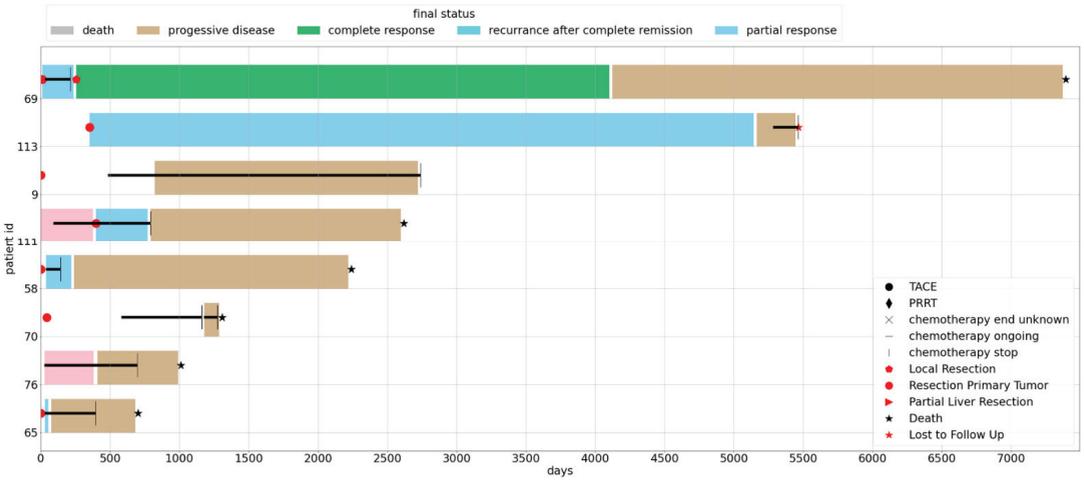
(**d**)

Figure 3. *Cont.*
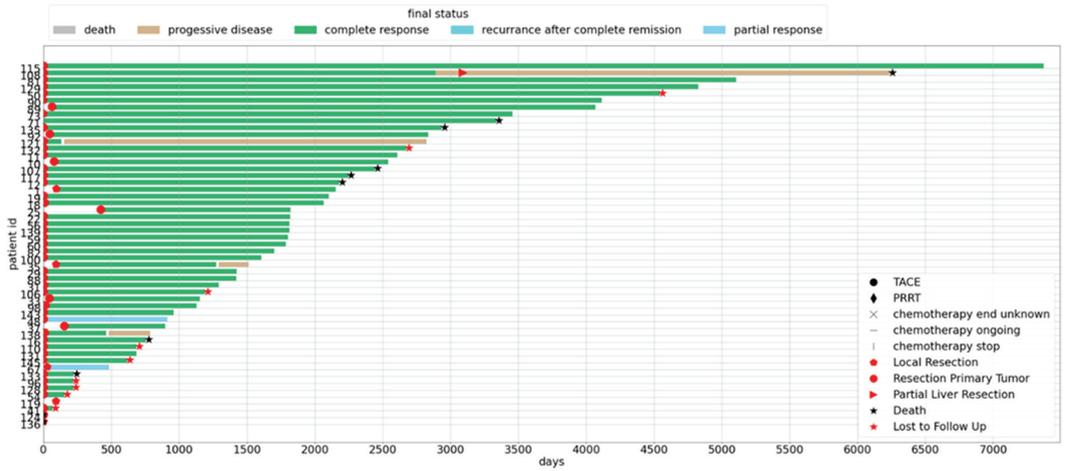
(**e**)
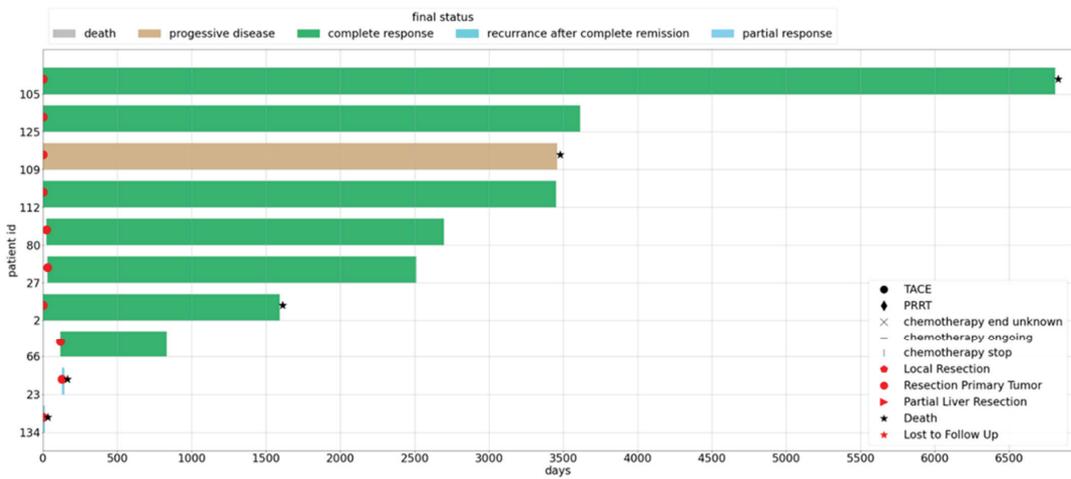
(**f**)

Figure 3. (**a**): Swimmer plot, group 1: patients (*n* = 32) treated with SSA alone; colors denote the final status (details in the figure legend), symbols denote different treatments. (**b**): Swimmer plot, group 2: patients (*n* = 26) treated with SSA parallel with TACE and/or PRRT; colors denote the final status, symbols denote different treatments. (**c**): Swimmer plot, group 3: patients (*n* = 8) treated with SSA followed by PRRT; colors denote the final status, symbols denote different treatments. (**d**): Swimmer plot, group 4: patients (*n* = 8) treated with chemotherapy; colors denote the final status, symbols denote different treatments. (**e**): Swimmer plot, group 5: patients (*n* = 53) not metastasized at diagnosis, local resection; colors denote the final status, symbols denote different treatments. (**f**): Swimmer plot, group 6: patients (*n* = 10) metastasized at diagnosis, surgery only; colors denote the final status, symbols denote different treatments.

When comparing survival in the different cohorts that included stage IV patients, five-year overall survival probability was 63.8% in group 1, 62.4% in group 2, 66.7% in group 3, 42.2% in group 4 and 66.7% in group 6 (Figure 4). Survival probability shown

as Kaplan–Meier curves of groups 1, 2, 3, 4 and 6 are shown in Figure 5. Hazard ratios for survival with reference group 2 (SSA only) were 1.49 ($p = 0.467$) for group 1, 0.715 ($p = 0.693$) for group 3, 2.34 ($p = 0.191$) for group 4, and 1.07 ($p = 0.920$) for group 6.
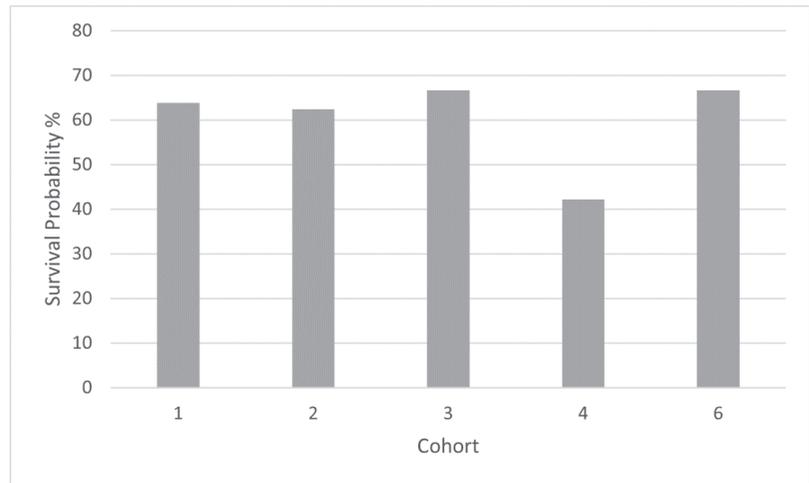
Figure 4. Five-year overall survival probability, groups 1–4 and 6 (metastasized patients) as percentages.
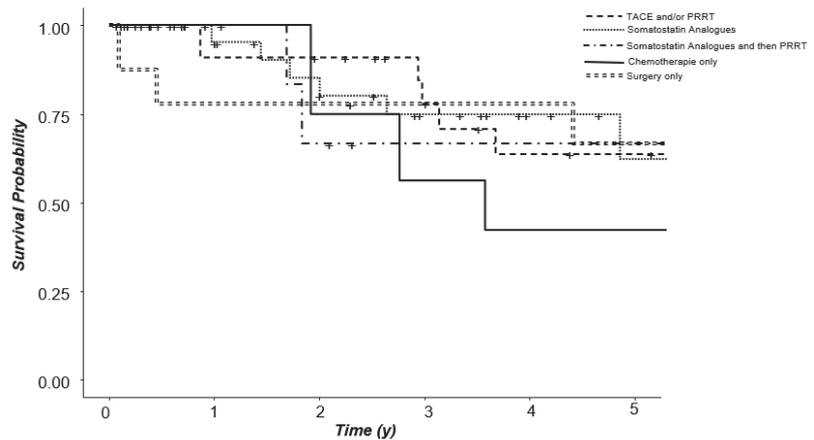
Figure 5. Survival probability, Kaplan–Meier analysis; reference group 2 (SSA only).

3.3. Survival According to Ki67 Index

The choice of treatment for siNET patients depends, among others, on the Ki67 index. Therefore, we also analyzed survival according to the respective grading (the WHO classification of 2010). An exact Ki67 staining result was available for 107/145 (73.8%) patients; grading (partly without an exact Ki67 staining result) was available for 129/145 (89%) patients. Five-year overall survival of patients with a G1 tumor ($n = 84$) was 77.9% and, thus, longer if compared to 54.2% in patients with G2 ($n = 43$) or G3 ($n = 2$) tumors (HR: 2.23; $p = 0.061$; Figure 6a). Progression-free survival rate in patients with G1 tumors was 45.4% significantly longer as compared to 21.8% in patients with a G2 or G3 tumor (HR: 2.06; $p = 0.015$; Figure 6b).

(**a**)

(**b**)
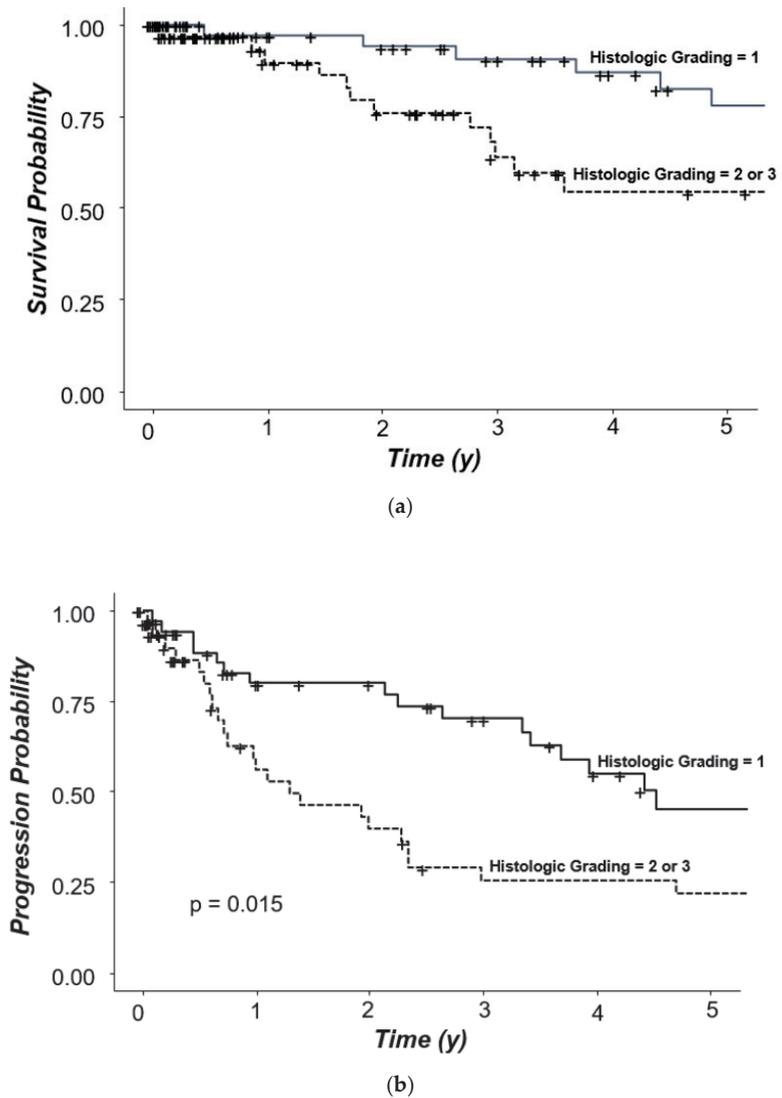
Figure 6. (**a**): Survival probability according to histological grading, all patients. (**b**): Progression probability according to histological grading, all patients.

3.4. Survival According to the Resection of Primary Tumor

In siNET, the resection of the primary tumor might be of benefit for the patients also in a metastasized stage. Therefore, we also analyzed survival in patients with or without resected primary tumor. Five-year overall survival of patients whose primary tumor was resected (*n* = 125) was 73.1% and, thus, significantly longer if compared to 33.3% in patients without a resection of their primary tumor (HR: 4.31; *p* = 0.003; Figure 7a). Three-year progression-free survival of patients whose primary tumor was resected was 41.3% vs. 22.2% in non-resected patients and, thus, significantly longer (Figure 7b; HR: 2.27 *p* = 0.041).
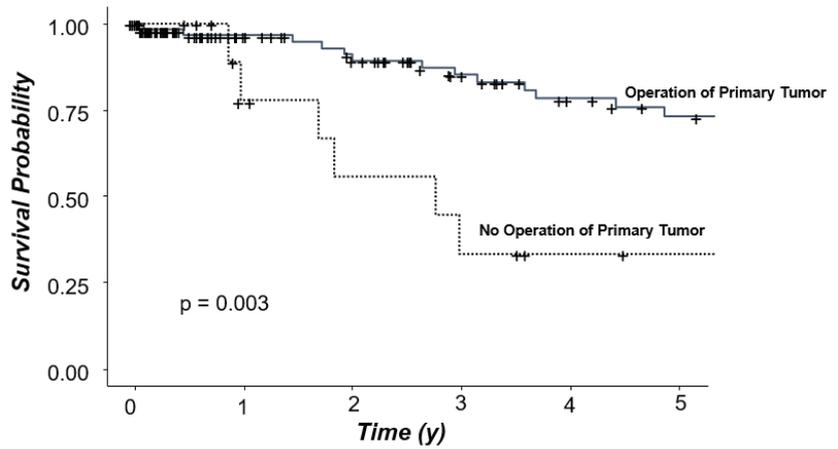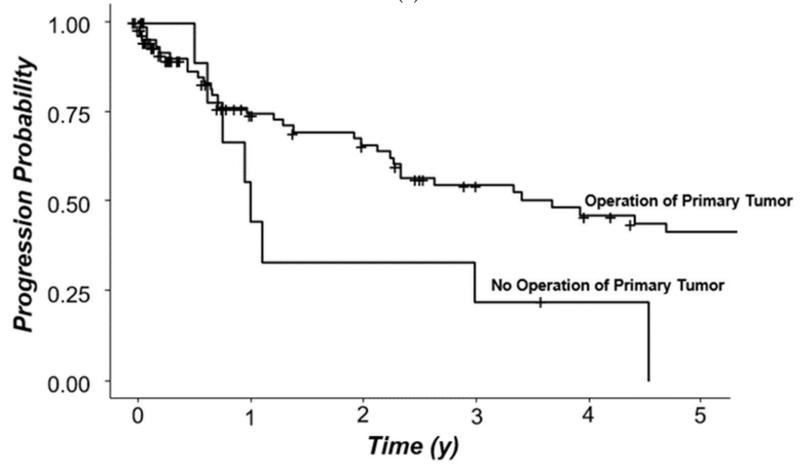
Figure 7. (**a**): Survival probability according to resection of the primary tumor, all patients. (**b**): Progression probability according to resection of the primary tumor, all patients.

4. Discussion

In this large cohort of patients, we analyzed survival according to treatment in patients with siNET, a rare tumor and orphan disease. We found that, in line with the literature, patients with low-grade NET had a better survival than patients with intermediate- or high-grade tumors, and that patients benefitted from a resection of their primary tumor [22–25].

However, when analyzing survival according to treatment regardless of the Ki67 index, the picture is quite diverse. Patients often underwent long-term treatments with prolonged phases without any therapy. Furthermore, for most patients, there was no clear path as other malignant diseases with, e.g., first treatment A, followed by treatment B and so forth. Instead, most patients received different treatments such as locoregional (TACE) or systemic treatments in different order. Many patients underwent surgery for localized or metastatic disease at some point of time, and most received SSA treatment. Taken together, we decided to split the whole cohort in different subgroups according to the predominant therapeutic regimen and visualized each patient's pathway by a line in a classical swimmer plot. One limitation of the study clearly is that, due to the retrospective design and the large

time span, a clear division of all patients was not possible. Therefore, we chose based on the predominant treatment the patient received for the majority of time. Similar analyses have been published from the Swiss NET registry but without survival analyses [26].

Most metastasized patients in our cohort were treated with a combination of SSA and locoregional treatments, either PRRT or TACE (for liver-dominant tumors) or both. SSA followed by PRRT upon progression was administered to eight patients only. This approach was recently prospectively investigated in the NETTER-1 trial [11], showing a clear survival benefit for patients with disease progression treated with PRRT over patients with dose escalation of the SSA (PFS: NR vs. 8.4 months; $p > 0.001$; HR 0.21).

Interestingly, the five-year survival probability in our cohort was comparable between all groups except for the patients that received chemotherapy who showed a shorter survival time, which was also observed by Faggiano et al. in a mixed cohort of G1/2 NET patients [27]. They split up a cohort of 99 patients with ≥ 2 lines of treatment into four different groups according to the therapeutic sequence, and analyzed PFS of first- and second-line treatment. Interestingly, there was no significant difference in either line for each group in this retrospective multicenter analysis. Toxicity, however, was higher in patients receiving either chemotherapy or everolimus. Chemotherapy in siNET is mainly administered in patients with G2 or G2 tumors with a Ki67 > 20%. In our cohort, there were only two patients with a G3 tumor; the remaining patients were G1/2. However, due to the low number, no clear interpretation is possible here.

The role of cytotoxic chemotherapy in siNET patients is still debatable since only small series of patients with GEP-NET that received chemotherapy are described in the literature, often mixed groups of patients with different primary tumors or combination treatments [28].

Shortcomings of our study are the retrospective and single-center nature, although we were able to analyze a large data set over a long period. However, due to possibly incomplete data and difficult comparability of different subgroups as outlined above, survival analyses have to be interpreted with caution.

Taken together, we show in this retrospective trial that treatment of siNET is rarely carried out in a strictly linear manner and involves several therapeutic options. Survival, however, is comparable between the groups and depends mainly on the resection of the primary tumor and grading.

References

1. Oronsky, B.; Ma, P.C.; Morgensztern, D.; Carter, C.A. Nothing But NET: A Review of Neuroendocrine Tumors and Carcinomas. *Neoplasia* **2017**, *19*, 991–1002. [CrossRef] [PubMed]

2. Van Der Zwan, J.M.; Trama, A.; Otter, R.; Larrañaga, N.; Tavilla, A.; Marcos-Gragera, R.; Dei Tos, A.P.; Baudin, E.; Poston, G.; Links, T. Rare neuroendocrine tumours: Results of the surveillance of rare cancers in Europe project. *Eur. J. Cancer* **2013**, *49*, 2565–2578. [CrossRef] [PubMed]

3. Rindi, G.; Klersy, C.; Albarello, L.; Baudin, E.; Bianchi, A.; Buchler, M.W.; Caplin, M.; Couvelard, A.; Cros, J.; de Herder, W.W.; et al. Competitive Testing of the WHO 2010 versus the WHO 2017 Grading of Pancreatic Neuroendocrine Neoplasms: Data from a Large International Cohort Study. *Neuroendocrinology* **2018**, *107*, 375–386. Available online: https://www.karger.com/Article/FullText/494355 (accessed on 26 January 2020). [CrossRef] [PubMed]

4. Graham, M.M.; Gu, X.; Ginader, T.; Breheny, P.; Sunderland, J. 68Ga-DOTATOC imaging of neuroendocrine tumors: A systematic review and metaanalysis. *J. Nucl. Med.* **2017**, *58*, 1452–1458. [CrossRef]

5. Bozkurt, M.F.; Virgolini, I.; Balogova, S.; Beheshti, M.; Rubello, D.; Decristoforo, C.; Ambrosini, V.; Kjaer, A.; Delgado-Bolton, R.; Kunikowska, J.; et al. Guideline for PET/CT imaging of neuroendocrine neoplasms with 68Ga-DOTA-conjugated somatostatin receptor targeting peptides and 18F–DOPA. *Eur. J. Nucl. Med. Mol. Imaging* **2017**, *44*, 1588–1601. [CrossRef] [PubMed]

6. Poeppel, T.D.; Binse, I.; Petersenn, S.; Lahner, H.; Schott, M.; Antoch, G.; Brandau, W.; Bockisch, A.; Boy, C. 68Ga-DOTATOC versus 68Ga-DOTATATE PET/CT in functional imaging of neuroendocrine tumors. *J. Nucl. Med.* **2011**, *52*, 1864–1870. [CrossRef] [PubMed]

7. Strosberg, J.R.; Halfdanarson, T.R.; Bellizzi, A.M.; Chan, J.A.; Dillon, J.; Heaney, A.P.; Kunz, P.L.; O'Dorisio, T.M.; Salem, R.; Segelov, E.; et al. The north American neuroendocrine tumor society consensus guidelines for surveillance and medical management of midgut neuroendocrine tumors. *Pancreas* **2017**, *46*, 707–714. [CrossRef]

8. Howe, J.R.; Cardona, K.; Fraker, D.L.; Kebebew, E.; Untch, B.R.; Wang, Y.Z.; Law, C.H.; Liu, E.H.; Kim, M.K.; Menda, Y.; et al. The surgical management of small bowel neuroendocrine tumors. *Pancreas.* **2017**, *46*, 715–731. [CrossRef]

9. Caplin, M.E.; Pavel, M.; Cwikla, J.B.; Phan, A.T.; Raderer, M.; Sedlackova, E.; Cadiot, G.; Wolin, E.M.; Capdevila, J.; Wall, L.; et al. Anti-tumour effects of lanreotide for pancreatic and intestinal neuroendocrine tumours: The CLARINET open-label extension study. *Endocrine-Related Cancer* **2016**, *23*, 191–199. [CrossRef]

10. Rinke, A.; Müller, H.-H.; Schade-Brittinger, C.; Klose, K.-J.; Barth, P.; Wied, M.; Mayer, C.; Aminossadati, B.; Pape, U.-F.; Bläker, M.; et al. Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: A report from the PROMID study group. *J. Clin. Oncol.* **2009**, *27*, 4656–4663. [CrossRef]

11. Strosberg, J.; El-Haddad, G.; Wolin, E.; Hendifar, A.; Yao, J.; Chasen, B.; Mittra, E.; Kunz, P.L.; Kulke, M.H.; Jacene, H.; et al. Phase 3 trial of 177lu-dotatate for midgut neuroendocrine tumors. *N. Engl. J. Med.* **2017**, *376*, 125–135. [CrossRef] [PubMed]

12. Fazio, N.; Buzzoni, R.; Fave, G.D.; Tesselaar, M.E.; Wolin, E.; Van Cutsem, E.; Tomassetti, P.; Strosberg, J.; Voi, M.; Bubuteishvili-Pacaud, L.; et al. Everolimus in advanced, progressive, well-differentiated, non-functional neuroendocrine tumors: RADIANT-4 lung subgroup analysis. *Cancer Sci.* **2017**, *109*, 174–181. Available online: http://doi.wiley.com/10.1111/cas.13427 (accessed on 1 June 2018). [CrossRef] [PubMed]

13. Yao, J.C.; Fazio, N.; Singh, S.; Buzzoni, R.; Carnaghi, C.; Wolin, E.; Tomasek, J.; Raderer, M.; Lahner, H.; Voi, M.; et al. Everolimus for the treatment of advanced, non-functional neuroendocrine tumours of the lung or gastrointestinal tract (RADIANT-4): A randomised, placebo-controlled, phase 3 study. *Lancet* **2015**, *387*, 968–977. [CrossRef]

14. Chatzellis, E.; Angelousi, A.; Daskalakis, K.; Tsoli, M.; Alexandraki, K.I.; Wachuła, E.; Meirovitz, A.; Maimon, O.; Grozinsky-Glasberg, S.; Gross, D.; et al. Activity and Safety of Standard and Prolonged Capecitabine/Temozolomide Administration in Patients with Advanced Neuroendocrine Neoplasms. *Neuroendocrinology* **2019**, *109*, 333–345. [CrossRef] [PubMed]

15. Ramirez, R.A.; Beyer, D.T.; Chauhan, A.; Boudreaux, J.P.; Wang, Y.-Z.; Woltering, E.A. The Role of Capecitabine/Temozolomide in Metastatic Neuroendocrine Tumors. *Oncologist* **2016**, *21*, 671–675. [CrossRef] [PubMed]

16. Welin, S.; Sorbye, H.; Sebjornsen, S.; Knappskog, S.; Busch, C.; Öberg, K. Clinical effect of temozolomide-based chemotherapy in poorly differentiated endocrine carcinoma after progression on first-line chemotherapy. *Cancer* **2011**, *117*, 4617–4622. [CrossRef]

17. Etoposide and Cisplatin Versus Irinotecan and Cisplatin as the First-Line Therapy for Patients with Advanced, Poorly Differentiated Gastroenteropancreatic Neuroendocrine Carcinoma: A Randomized Phase 2 Study-PubMed. Available online: https://pubmed.ncbi.nlm.nih.gov/32293725/?from_term=etoposid+neuroendocrine&from_sort=date&from_page=2&from_pos=3 (accessed on 11 June 2020).

18. Egger, M.E.; Armstrong, E.; Martin, R.C.; Scoggins, C.R.; Philips, P.; Shah, M.; Konda, B.; Dillhoff, M.; Pawlik, T.M.; Cloyd, J.M. Transarterial Chemoembolization vs Radioembolization for Neuroendocrine Liver Metastases: A Multi-Institutional Analysis. *J. Am. Coll. Surg.* **2020**, *230*, 363–370. [CrossRef] [PubMed]

19. Grozinsky-Glasberg, S.; Kaltsas, G.; Kaltsatou, M.; Lev-Cohain, N.; Klimov, A.; Vergadis, V.; Uri, I.; Bloom, A.I.; Gross, D.J. Hepatic intra-arterial therapies in metastatic neuroendocrine tumors: Lessons from clinical practice. *Endocrine* **2018**, *60*, 499–509. [CrossRef]

20. Van Den Heede, K.; Chidambaram, S.; Van Slycke, S.; Brusselaers, N.; Warfvinge, C.F.; Ohlsson, H.; Gustafsson, R.; Nordenström, E.; Almquist, M. Long-term survival of metastatic small intestine neuroendocrine tumors: A meta-analysis. *Endocr. Relat. Cancer* **2022**, *29*, 163–173. [CrossRef]

21. Koch, C.; Koca, E.; Filmann, N.; Husmann, G.; Bojunga, J. Time from first tumor manifestation to diagnosis in patients with GEP-NET: Results from a large German tertiary referral center. *Medicine* **2021**, *100*, e27276. [CrossRef]

22. Gangi, A.; Manguso, N.; Gong, J.; Crystal, J.S.; Paski, S.C.; Hendifar, A.E.; Tuli, R. Midgut Neuroendocrine Tumors with Liver-only Metastases: Benefit of Primary Tumor Resection. *Ann. Surg. Oncol.* **2021**, *27*, 4525–4532. [CrossRef] [PubMed]

23. Hallet, J.; Law, C.; Pasieka, J.; Koea, J.; Meyer-Rochow, W. Role of Primary Tumor Resection for Metastatic Small Bowel Neuroendocrine Tumors. *World J. Surg.* **2020**, *45*, 213–218. [CrossRef] [PubMed]

24. Polcz, M.; Schlegel, C.; Edwards, G.C.; Wang, F.; Tan, M.; Kiernan, C.; Solórzano, C.C.; Idrees, K.; Parikh, A.; Bailey, C.E. Primary Tumor Resection Offers Survival Benefit in Patients with Metastatic Midgut Neuroendocrine Tumors. *Ann. Surg. Oncol.* **2020**, *27*, 2795–2803. [CrossRef] [PubMed]

25. Tsilimigras, D.I.; Hyer, J.M.; Paredes, A.Z.; Ejaz, A.; Cloyd, J.M.; Beane, J.D.; Dillhoff, M.; Tsung, A.; Pawlik, T.M. Resection of Primary Gastrointestinal Neuroendocrine Tumor Among Patients with Non-Resected Metastases Is Associated with Improved Survival: A SEER-Medicare Analysis. *Gastrointest. Surg.* **2021**, *25*, 2368–2376. Available online: http://www.ncbi.nlm.nih.gov/pubmed/33403563 (accessed on 20 May 2021).

26. Kollar, A.; Bütikofer, L.; Ochsenbein, A.; Stettler, C.; Trepp, R. Treatment sequence in patients with neuroendocrine tumours: A nationwide multicentre, observational analysis of the Swiss neuroendocrine tumour registry. *Swiss Med Wkly.* **2020**, *150*, w20176. Available online: http://emh.ch/en/services/permissions.html (accessed on 20 May 2021). [CrossRef] [PubMed]

27. Faggiano, A.; Elios, O.B.O.; Di Maio, S.; Mocerino, C.; Ottaviano, M.; De Divitiis, C.; Guarnotta, V.; Dolce, P.; Modica, R.; Puliafito, I.; et al. Therapeutic sequences in patients with grade 1−2 neuroendocrine tumors (NET): An observational multicenter study from the ELIOS group. *Endocrine* **2019**, *66*, 417–424. Available online: https://pubmed.ncbi.nlm.nih.gov/30875056/ (accessed on 20 May 2021).

28. Ostwal, V.; Basu, S.; Bhargava, P.; Shah, M.; Parghane, R.V.; Srinivas, S.; Chaudhari, V.; Bhandare, M.S.; Shrikhande, S.V.; Ramaswamy, A. Capecitabine-Temozolomide (CAPTEM) in advanced Grade 2 and grade 3 Neuroendocrine neoplasms (NENs)-benefits of chemotherapy in NENs with significant 18FDG uptake. *Neuroendocrinology* **2021**, *111*, 998–1004. [CrossRef] [PubMed]

Article

Duodenal Gastric Metaplasia and Duodenal Neuroendocrine Neoplasms: More Than a Simple Coincidence?

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Abstract: Background: Duodenal gastric metaplasia (DGM) is considered a precancerous lesion. No data are available regarding its possible role as a risk factor for duodenal neuroendocrine neoplasms (dNENs). Aims: To assess the prevalence of DGM in a cohort of dNENs. Methods: Subgroup analysis of a retrospective study including dNEN patients who underwent surgical resection between 2000 and 2019 and were observed at eight Italian tertiary referral centers. Results: 109 dNEN patients were evaluated. Signs of DGM associated with the presence of dNEN were reported in 14 patients (12.8%). Among these patients, nine (64.4%) had a dNEN of the superior part of the duodenum, one (7.1%) a periampullary lesion, three (21.4%) a dNEN located in the second portion of the duodenum, with a different localization distribution compared to patients without DGM ($p = 0.0332$). Ten were G1, three G2, and in one patient the Ki67 was not available. In the group with DGM, six patients (35.7%) were classified at stage I, five (28.6%) at stage II, three (21.4%) at stage III, and no one at stage IV. In the group without DGM, 20 patients (31%) were at stage I, 15 (15%) at stage II, 42 (44%) at stage III, and 19 (20%) at stage IV ($p = 0.0236$). At the end of the study, three patients died because of disease progression. Conclusions: our findings might suggest that DGM could represent a feature associated with the occurrence of dNEN, especially for forms of the superior part of the duodenum, which should be kept in mind in the endoscopic follow up of patients with DGM. Interestingly, dNEN inside DGM showed a more favorable staging, with no patients in stage IV. The actual relationship and the clinical relevance of this possible association require further clarification.

Keywords: duodenal neuroendocrine neoplasms; duodenal gastric metaplasia; risk factor; epidemiology

1. Introduction

Duodenal neuroendocrine neoplasms (dNENs) are rare and heterogeneous tumors that represent up to 3% of all duodenal neoplasms [1]. They usually present in the 6th decade of age with a slight male predominance [2]. Duodenal NENs are usually well-differentiated neoplasms; however, they can be metastatic in up to 55% of cases [3]. Their natural history, clinical characteristics, biological mechanisms, medical or surgical treatment, and prognosis are still poorly understood.

Duodenal NENs originate from aberrant neuroendocrine duodenal cell proliferation; in this microenvironment, complex interactions take place. The recognition of the molecular mechanisms participating in neoplastic transformation could increase the challenging management of this disease. However, at present, little is known about the risk factors of these neoplasms.

The normal mucosa of the duodenum is composed of absorbing columnar enterocytes and secreting goblet cells. Duodenal gastric metaplasia (DGM) is characterized by the replacement of the normal duodenal epithelial cells with gastric mucus-secreting cells that resemble gastric foveolar epithelium. It is commonly considered a precancerous lesion often associated with chronic inflammation. It is generally the consequence of abnormally high production of gastric acid triggered by *Helicobacter Pylori* (HP) infection [4]. When hypersecretion reaches the duodenum, the enterocytes of the villi react with apical mucin metaplasia to mitigate the unwanted low pH of the microenvironment. Besides HP infection, DGM has been reported in association with other conditions, such as medications (i.e., nonsteroidal anti-inflammatory drugs; NSAIDs), celiac disease [5], and Crohn's disease involving the duodenum [6]. However, DGM has been described also in the absence of all these conditions, although its actual etiology in the latter group of patients is unclear. Furthermore, DGM usually disappears following HP eradication [7], whereas the natural course of DGM in celiac patients or patients without a recognized cause, even with the application of a strict gluten-free diet, is still poorly known.

It still remains a question of debate whether DGM could represent a neoplastic risk factor. A high frequency (40.5%) of DGM has been found in duodenal adenomas [8]. It might be possible that metaplasia precedes the neoplastic transformation as has been reported in other gastrointestinal malignancies including esophagus (intestinal metaplasia in Barrett's esophagus–dysplasia–carcinoma sequence [9] and stomach [10] and colorectal cancer [11]). Furthermore, DGM has been associated with genetic alterations, such as GNAS and KRAS mutations, which are involved in different types of tumors including duodenal adenocarcinoma.

However, no data are available regarding the possible role of DGM as a risk factor for the occurrence of dNEN. Taking into account these observations and the lack of clear-cut data regarding the natural history of dNEN, we aimed at assessing the prevalence of DGM in a cohort of dNENs. The secondary aim was to explore whether the presence of DGM had any impact on the characteristics or outcome of the current cohort of dNENs.

2. Materials and Methods

We performed a subgroup analysis of a retrospective study [3] including all consecutive patients with dNEN, who underwent surgical resection between 2000 and 2019 and who were observed at eight Italian tertiary referral centers.

All data were retrieved at the center where each patient had been diagnosed and followed up. Participating study centers sent the anonymized data of patients to the lead center. The study's inclusion criteria were age > 18 years, histological diagnosis of dNEN of any grade and stage, surgical treatment of the primary tumor, availability of complete histopathological examination of the surgical specimen, and clinical data with a minimum 3 month follow up after diagnosis. The exclusion criteria were histological findings of mixed neuroendocrine non-neuroendocrine neoplasms (MiNEN), age < 18 years, the use of experimental drugs during the 2 months preceding inclusion in this study, and pregnancy

or breastfeeding status. Due to the retrospective nature of this study, ethical approval was waived.

The tumor characteristics analyzed comprised the site and the size of the primary tumor, number of lesions, grade, and stage (i.e., localized, regional, distant, and unknown). The patient's characteristics included the age at first diagnosis, the presence of genetic syndrome (i.e., multiple endocrine neoplasia (MEN)-1), and the presence of functioning neoplasms.

Medical history data were collected and recorded by physicians in electronic health records, comprising the clinical history, age at diagnosis, treatments received, clinical and biochemical parameters, radiological imaging, endoscopy examinations, and nuclear medicine imaging were recorded and evaluated at each referral center. The type of surgical intervention was recorded for all the patients.

Neoplasms were classified according to the WHO 2019 classification [12] and staged according to the current European Neuroendocrine Tumor Society (ENETS) TNM clinical staging [13].

For each included patient, the endoscopic or surgical specimen and related histopathological data were assessed to verify the presence or absence of DGM. Concomitant treatment with proton pump inhibitors (PPIs) was recorded.

Statistical Analysis

Descriptive statistics were used to summarize the data. Continuous variables with normal distribution were expressed as the median (i.e., range); categorical variables were reported as the count (i.e., percentage). All data were tested for distribution normality by the Kolmogorov–Smirnoff test. The differences between groups were assessed with the Mann–Whitney test and the Kruskal–Wallis test as appropriate. Comparisons between groups were assessed using the χ^2 test or Fisher's exact test. The analyses were carried out using GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA, USA).

3. Results

From 2000 to 2019, 109 patients with histologically confirmed dNEN were included in the study as previously reported (Figure 1).
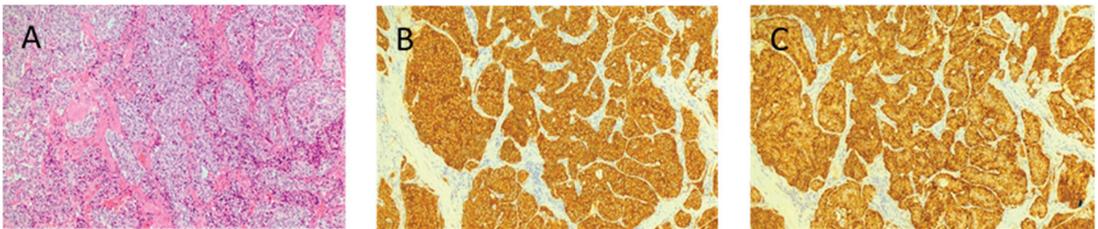
Figure 1. Representative hematoxylin and eosin stain (**A**), synaptophysin (**B**) and chromogranin (**C**) of a duodenal NEN in a 75 year old male patient. The neoplasia was characterized by low mitotic activity (MIB1-labeling index: 0.2%, mitotic index: 0), and a final diagnosis of G1 neuroendocrine tumor was reached (original magnifications: 40×).

The DGM associated with a dNEN was reported in 14 patients (12.8%). None of these patients had a concomitant HP infection, celiac disease, or Crohn's disease. Concomitant use of NSAIDs was excluded for all 14 patients.

The baseline characteristics of these 14 patients were compared to the clinical features of the remaining 95 patients without signs of DGM (Table 1). We observed a male prevalence in both groups, whereas the patients with DGM were older (61.5 versus 58 years old), even if this difference was not statistically significant. In the two groups, the median diameter of the neoplasms was similar (being quite small, namely, 15 in patients without DGM and

11 mm in patients with DGM), and the majority of tumors were single. Location of the primary NEN was significantly different between the two groups (*p* = 0.0332): among the 14 patients with DGM, 9 had a dNEN of the superior part of the duodenum (64.4%), 1 had a periampullary neoplasm (7.1%), in 3, the dNEN was located in the second portion of the duodenum (21.4%), whereas in 1 patient the location was not specified. Among the 95 patients with dNEN without DGM, the majority (42.1%) showed periampullary tumors.

Table 1. Baseline characteristics of patients with duodenal gastric metaplasia (DGM) associated with duodenal neuroendocrine neoplasms (dNENs) compared to dNEN patients without DGM.

Characteristics	dNENs		*p*
	w/o DGM n (%)	with DGM n (%)	
Number of patients	95 (87)	14 (13)	
Age (years), median (range)	58 (17–83)	61.5 (32–74)	n.s.
Gender (M/F)	57/38	(11/3)	n.s.
Location			
Superior part of the duodenum	27 (28.4)	9 (64.4)	
Periampullary	40 (42.1)	1 (7.1)	0.0332
Descending duodenum	21 (22.1)	3 (21.4)	
NA	7 (7.4)	1 (7.1)	
Grading (12)			
G1	56 (58.9)	10 (71.5)	
G2	23 (24.3)	3 (21.4)	n.s.
G3	7 (7.3)	0	
NA	9 (9.5)	1 (7.1)	
Diameter (mm), median (range)	15 (1.5–130)	11 (3–37)	n.s.
Functioning (gastrinoma/somatostatinoma) Nonfunctioning	28 (29.4) (23/4) 69 (70.6)	5 (35.7) (4/1) 9 (64.3)	n.s.
Single Multiple	82 (86.3) 13 (13.7)	11 (78.6) 3 (21.4)	n.s.
Stage (13)			
I	20 (21)	6 (42.8)	
II	15 (15)	5 (35.7)	0.0236
III	42 (44)	3(21.4)	
IV	19 (20)	0	
Type of surgery			
Pancreaticoduodenectomy	58 (61)	3 (21.4)	
Total pancreatectomy	4 (4.2)	0	
Duodenotomy + enucleation	28 (29.5)	6 (42.9)	0.0007
Partial duodenectomy + lymphadenectomy	5 (5.3)	5 (35.7)	
MEN-1	17 (17.9)	1 (7.1)	n.s.
Proton pump inhibitor	31 (32.6)	5 (35.7)	n.s.

As concerning grading, among the patients with DGM, 10 were G1; 3 G2; while in 1 patient the ki67 was not specified. None of the tumors inside DGM was a poorly differentiated neoplasm. Among the 95 patients without DGM, 56 were G1; 23 G2; 7 G3; whereas in 9 patients the Ki67 was not available.

The staging had a significantly different distribution between the two groups (*p* = 0.0236); in the group with DGM, six patients were classified as stage I; five as stage II; three as stage III; no one at stage IV. In the other group without DGM, 20 patients were at stage I; 15 at stage II; 42 at stage III; 19 at stage IV.

The type of surgery was significantly different between the two groups (*p* = 0.0007): 3 out of the 14 patients (21.5%) with DGM underwent pancreaticoduodenectomy, 6 (42.8%) duodenotomy with enucleation, and 5 (35.7%) partial duodenectomy and lymphadenectomy. Among the 95 patients without DGM, 58 (61%) underwent pancreaticoduodenectomy, 4 (4.2%) total pancreatectomy, 28 (29.5%) duodenotomy and enucleation, and five (5.3%) partial duodenectomy and lymphadenectomy.

In the group of 14 patients with DGM, the 5 patients at stage III presented with lymph node metastases at diagnosis and received treatment with somatostatin analogs (SSAs), which were continued after surgery.

One patient out of 14 (7.1%) with DGM-associated dNEN and 17 out of 95 (17.9%) with dNEN not associated with DGM were diagnosed with MEN-1 syndrome, without any significant difference in the percentage of MEN-1. In both groups, the majority of the tumors were nonfunctioning. Five patients (35.7%) were treated with proton pump inhibitors (PPIs) versus 31 patients in the group without DGM (32.6%).

At the end of the study, three patients out of the 14 with DGM (21.4%) were dead, of which only one was due to the fact of disease progression (occurrence of distant liver metastases treated with SSA and chemotherapy). In the group without DGM, 18 patients passed away (18.9%), 13 due to the fact of disease progression.

4. Discussion

Duodenal NENs are rare neoplastic lesions born by the aberrant proliferation of the neuroendocrine epithelial cells of the duodenal mucosa [3]. To date, no specific risk factors for the development of dNEN are known; thus, more efforts should be made to identify patients at risk (i.e., by the identification of preneoplastic lesions) in order to develop disease-specific surveillance [14]. In our multicenter study, we demonstrated that the existence of a DGM characterized a non-negligible percentage of dNEN cases, suggesting this could represent a potential risk factor for dNEN. DGM was, in fact, found in almost 13% of the entire cohort of 109 dNEN patients surgically treated.

However, the actual percentage of DGM in the general population is poorly known as variable percentages have been reported in the literature [15,16], and this might be worthy of investigation.

The percentage reported in the current paper was, conversely, quite far from the high percentage described for duodenal adenomas in which DGM has been found to be as high as 40.5%, even if this percentage could be underestimated, considering this alteration has never been described in relation with dNENs; therefore, one can hypothesize that with increasing awareness, this finding could have a greater frequency.

Many studies have demonstrated that several lesions that were thought to be metaplastic may actually represent a potential precursor of common neoplasms. For example, colorectal hyperplastic polyps, which exhibit preserved overall crypt organization and no epithelial dysplasia [17], are commonly considered potential precursors of colorectal cancer [18]; similarly, pancreatic intraepithelial neoplasia 1A, which has also been previously regarded as mucinous metaplasia, is now well known to be the earliest stage precursor of invasive pancreatic adenocarcinoma [19]. Likewise, some duodenal tumors, particularly those with a gastric epithelial phenotype, were interestingly proven to arise from DGM [20,21]. DGM is a condition characterized by the metaplastic replacement of the normal duodenal enterocytes by mucinous PAS-positive cells, migrating from the Brunner's gland ducts and resembling the superficial gastric foveolar epithelium [22]. To be accurate, DGM should be distinguished from duodenal gastric heterotopia (DGH), which is instead characterized by the presence of both the gastric foveolar epithelium and the oxyntic glands. Because of its fully organized structure, DGH has been interpreted as a congenital lesion [23], while DGM is generally regarded as an acquired reactive process caused by chronic inflammatory conditions [24]. The prevalence of DGM is, in fact, higher in patients with HP infection, as it induces a high level of acid burden in the duodenum by increasing gastrin secretion; moreover, the presence of DGM may create a suitable

environment for HP colonization, which may exert a cytotoxic effect on mucosal cells and, thus, to the development of further DGM [24,25]. In our study, none of the patients had a concomitant HP infection. As concerned PPIs, five patients in our cohort with DGM were taking PPIs, a fraction not different from the group without DGM, without therefore suggesting a particular etiopathogenetic role of PPIs in the genesis of DGM-related dNEN. However, even if this percentage was not different between the groups, it was surely of relevance in both groups; therefore, one could also hypothesize that PPIs could have a role in the development of duodenal NENs. Unfortunately, this study did not have the power to investigate this topic.

Concerning the possible different characteristics or outcomes of the dNENs arising in DGM, when comparing the two groups, with and without DGM, we observed that the 14 patients with DGM were younger, and most of the dNENs with GDM were located in the superior part of the duodenum. The reason for this is unknown. It could be hypothesized that there are some different etiopathogenetic factors in the genesis of dNEN originating from the first duodenal portion (for example, the effect of hydrochloric acid or different distributions of neuroendocrine cells types, i.e., somatostatin-, gastrin-, serotonin-producing cells). Unfortunately, these are only speculative hypotheses, and this type of study cannot answer this question. Moreover, interestingly, among the 14 patients with DGM, none showed a metastatic disease (none at stage IV) or G3 neoplasms. This might suggest that dNEN associated with DGM could be more similar to the gastric neuroendocrine neoplasms, such as those arising from gastric metaplasia and, therefore, more indolent and lower grade.

Genetic mutations have been also demonstrated to play a potential role in the development of DGM; GNAS and KRAS mutations, for instance, which are generally frequently present in benign/low-grade tumors of the digestive tract [18,26–28], were reported to be prevalent in DGM lesions, suggesting that these genetic alterations induce the proliferation of metaplastic epithelium [29]. Given these demonstrations and based on the association observed in our study, one might speculate that the occurrence of DGM is an epiphenomenon of genetic mutations and a chronic inflamed microenvironment [22] together with the gastrin-mediated dysregulation of molecular pathways [4,25–27], promoting tumorigenesis, including dNEN formation [28], with possible implications for the endoscopic follow-up of patients with DGM. In the presence of DGM at histology, in fact, it might be possible to consider a closer endoscopic follow up in order to detect early the presence of dNEN.

We acknowledge two main limitations of our study. First, the retrospective nature of the study and the small sample of patients limit the strength of our conclusions; however, dNEN is a rare disease; thus, large prospective cohort studies are difficult. Second, the histological revision of the pathologic samples was not centralized. However, only pathological examinations performed at referral centers for NENs were included in the study, whereas patients with incomplete information were excluded from the analysis.

5. Conclusions

In summary, DGM was found in almost 13% of the entire cohort of 109 dNEN patients surgically treated, thus representing a remarkable percentage. Given these data, one might speculate that the presence of DGM could precede the development of dNEN; the common finding of this lesion in the general population as well as the current lack of disease-specific literature allow for the clinical relevance of this possible association to be clarified; however, it should be kept in mind in the endoscopic follow up of patients with DGM that even the lack of clear-cut evidence does not allow to suggest a specific timeline for endoscopic follow up. Moreover, the DGM-related dNEN could have a different natural history compared to the dNEN not related to DGM and, therefore, be susceptible to different treatments. In conclusion, our observations highlight the need for further studies, ideally creating international disease registries, to better understand the biology and natural history of dNEN and, thus, to improve the management of this heterogeneous disease.

References

1. Fitzgerald, T.L.; Dennis, S.O.; Kachare, S.D.; Vohra, N.A.; Zervos, E.E. Increasing Incidence of Duodenal Neuroendocrine Tumors: Incidental Discovery of Indolent Disease? *Surgery* **2015**, *158*, 466–471. [CrossRef] [PubMed]
2. Yao, J.C.; Hassan, M.M.; Phan, A.T.; Dagohoy, C.G.; Leary, C.C.; Mares, J.E.; Abdalla, E.K.; Fleming, J.B.; Vauthey, J.-N.; Rashid, A.; et al. One Hundred Years after "Carcinoid": Epidemiology of and Prognostic Factors for Neuroendocrine Tumors in 35,825 Cases in the United States. *J. Clin. Oncol.* **2008**, *26*, 3063–3072. [CrossRef] [PubMed]
3. Massironi, S.; Campana, D.; Partelli, S.; Panzuto, F.; Rossi, R.E.; Faggiano, A.; Brighi, N.; Falconi, M.; Rinzivillo, M.; Fave, G.D.; et al. Heterogeneity of Duodenal Neuroendocrine Tumors: An Italian Multi-Center Experience. *Ann. Surg. Oncol.* **2018**, *25*, 3200–3206. [CrossRef] [PubMed]
4. Harris, A.W.; Gummett, P.A.; Walker, M.M.; Misiewicz, J.J.; Baron, J.H. Relation between Gastric Acid Output, Helicobacter Pylori, and Gastric Metaplasia in the Duodenal Bulb. *Gut* **1996**, *39*, 513–520. [CrossRef] [PubMed]
5. Shaoul, R.; Marcon, M.A.; Okada, Y.; Cutz, E.; Forstner, G. Gastric Metaplasia: A Frequently Overlooked Feature of Duodenal Biopsy Specimens in Untreated Celiac Disease. *J. Pediatr. Gastroenterol. Nutr.* **2000**, *30*, 397–403. [CrossRef] [PubMed]
6. Kushima, R.; Borchard, F.; Hattori, T. A New Aspect of Gastric Metaplasia in Crohn's Disease: Bidirectional (Foveolar and Pyloric) Differentiation in So-Called 'Pyloric Metaplasia' in the Ileum. *Pathol. Int.* **1997**, *47*, 416–419. [CrossRef]
7. Ciancio, G.; Nuti, M.; Orsini, B.; Lovi, F.; Ortolani, M.; Palomba, A.; Amorosi, A.; Surrenti, E.; Milani, S. Regression of Duodenal Gastric Metaplasia in Helicobacter Pylori Positive Patients with Duodenal Ulcer Disease. *Dig. Liver Dis.* **2002**, *34*, 16–21. [CrossRef]
8. Rubio, A.C. Gastric Duodenal Metaplasia in Duodenal Adenomas. *J. Clin. Pathol.* **2007**, *60*, 661–663. [CrossRef]
9. Rubio, A.C.; Lagergren, J. Histological Features Pertinent to Local Tumour Progression in Barrett's Adenocarcinoma. *Anticancer Res.* **2003**, *23*, 3015–3018.
10. Rubio, A.C.; Jónasson, J.; Nesi, G.; Mandai, K.; Pisano, R.; King, A.; Owen, D. Extensive Intestinal Metaplasia in Gastric Carcinoma and in Other Lesions Requiring Surgery. A Study of 3421 Gastrectomy Specimens from Dwellers of the Atlantic and Pacific Basins. *J. Clin. Pathol.* **2005**, *58*, 1271–1277. [CrossRef]
11. Snover, D.C.; Jass, J.R.; Fenoglio-Preiser, C.; Batts, K.P. Serrated Polyps of the Large Intestine: A Morphologic and Molecular Review of an Evolving Concept. *Am. J. Clin. Pathol.* **2005**, *124*, 380–391. [CrossRef] [PubMed]
12. Nagtegaal, I.D.; Odze, R.D.; Klimstra, D.; Paradis, V.; Rugge, M.; Schirmacher, P.; Washington, K.M.; Carneiro, F.; Cree, I.A.; the WHO Classification of Tumours Editorial Board. The 2019 WHO Classification of Tumours of the Digestive System. *Histopathology* **2020**, *76*, 182–188. [CrossRef] [PubMed]
13. Rindi, G.; Klöppel, G.; Alhman, H.; Caplin, M.; Couvelard, A.; de Herder, W.W.; Erikssson, B.; Falchetti, A.; Falconi, M.; Komminoth, P.; et al. TNM Staging of Foregut (Neuro)Endocrine Tumors: A Consensus Proposal Including a Grading System. *Virchows Arch.* **2006**, *449*, 395–401. [CrossRef] [PubMed]
14. Rossi, R.E.; Rausa, E.; Cavalcoli, F.; Conte, D.; Massironi, S. Duodenal Neuroendocrine Neoplasms: A Still Poorly Recognized Clinical Entity. *Scand. J. Gastroenterol.* **2018**, *53*, 835–842. [CrossRef]
15. Seo, J.H.; Do, H.J.; Park, C.H.; Woo, H.O.; Youn, H.S.; Ko, G.H.; Baik, S.C.; Lee, W.K.; Cho, M.J.; Rhee, K.H.; et al. Helicobacter Pylori Infection and Duodenal Gastric Metaplasia in Healthy Young Adults. *Korean J. Gastroenterol.* **2013**, *61*, 191–195. Available online: https://pubmed.ncbi.nlm.nih.gov/23624732/ (accessed on 12 March 2022). [CrossRef]
16. Fitzgibbons, P.L.; Dooley, C.P.; Cohen, H.; Appleman, M.D. Prevalence of Gastric Metaplasia, Inflammation, and CampylObacter Pylori in the Duodenum of Members of a Normal Population. *Am. J. Clin. Pathol.* **1988**, *90*, 711–714. [CrossRef]
17. Teoh, H.H.; Delahunt, B.; Isbister, W.H. Dysplastic and Malignant Areas in Hyperplastic Polyps of the Large Intestine. *Pathology* **1989**, *21*, 138–142. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0031302516364984 (accessed on 26 February 2022). [CrossRef]

18. Beach, R.; Chan, A.O.-O.; Wu, T.-T.; White, J.A.; Morris, J.S.; Lunagomez, S.; Broaddus, R.R.; Issa, J.-P.J.; Hamilton, S.R.; Rashid, A. BRAF Mutations in Aberrant Crypt Foci and Hyperplastic Polyposis. *Am. J. Pathol.* **2005**, *166*, 1069–1075. [CrossRef]

19. Hruban, R.H.; Adsay, N.V.; Albores–Saavedra, J.; Compton, C.; Garrett, E.S.; Goodman, S.N.; Kern, S.E.; Klimstra, D.S.; Klöppel, G.; Longnecker, D.S.; et al. Pancreatic Intraepithelial Neoplasia. *Am. J. Surg. Pathol.* **2001**, *25*, 579–586. [CrossRef]

20. Kushima, R.; Rüthlein, H.J.; Stolte, M.; Bamba, M.; Hattori, T.; Borchard, F. 'Pyloric Gland-Type Adenoma' Arising in Heterotopic Gastric Mucosa of the Duodenum, with Dysplastic Progression of the Gastric Type. *Virchows Arch.* **1999**, *435*, 452–457. [CrossRef]

21. Ushiku, T.; Arnason, T.; Fukayama, M.; Lauwers, G.Y. Extra-Ampullary Duodenal Adenocarcinoma. *Am. J. Surg. Pathol.* **2014**, *38*, 1484–1493. [CrossRef] [PubMed]

22. Serra, S.; Jani, A.P. An Approach to Duodenal Biopsies. *J. Clin. Pathol.* **2006**, *59*, 1133–1150. [CrossRef] [PubMed]

23. Terada, T. Heterotopic Gastric Mucosa of the Gastrointestinal Tract: A Histopathologic Study of 158 Cases. *Pathol.-Res. Pract.* **2011**, *207*, 148–150. [CrossRef] [PubMed]

24. Shousha, S.; Spiller, R.; Parkins, R. The Endoscopically Abnormal Duodenum in Patients with Dyspepsia: Biopsy Findings in 60 Cases. *Histopathology* **1983**, *7*, 23–34. [CrossRef]

25. Satoh, K.; Kimura, K.; Yoshida, Y.; Ota, Y.; Taniguchi, Y.; Kihira, K.; Kasano, T.; Takimoto, T.; Suzuki, T.; Seki, M. Relationship between Helicobacter Pylori and Atrophic Gastritis. *Eur. J. Gastroenterol. Hepatol.* **1994**, *6*, S85–S858.

26. Furukawa, T.; Kuboki, Y.; Tanji, E.; Yoshida, S.; Hatori, T.; Yamamoto, M.; Shibata, N.; Shimizu, K.; Kamatani, N.; Shiratori, K. Whole-Exome Sequencing Uncovers Frequent GNAS Mutations in Intraductal Papillary Mucinous Neoplasms of the Pancreas. *Sci. Rep.* **2011**, *1*, 161. [CrossRef]

27. Yamada, M.; Sekine, S.; Ogawa, R.; Taniguchi, H.; Kushima, R.; Tsuda, H.; Kanai, Y. Frequent Activating GNAS Mutations in Villous Adenoma of the Colorectum. *J. Pathol.* **2012**, *228*, 113–118. [CrossRef]

28. Wu, J.; Matthaei, H.; Maitra, A.; Dal Molin, M.; Wood, L.D.; Eshleman, J.R.; Goggins, M.; Canto, M.I.; Schulick, R.D.; Edil, B.H.; et al. Recurrent GNAS Mutations Define an Unexpected Pathway for Pancreatic Cyst Development. *Sci. Transl. Med.* **2011**, *3*, 92ra66. [CrossRef]

29. Matsubara, A.; Ogawa, R.; Suzuki, H.; Oda, I.; Taniguchi, H.; Kanai, Y.; Kushima, R.; Sekine, S. Activating GNAS and KRAS Mutations in Gastric Foveolar Metaplasia, Gastric Heterotopia, and Adenocarcinoma of the Duodenum. *Br. J. Cancer* **2015**, *112*, 1398–1404. [CrossRef]

Article

Surgical Outcomes and Prognostic Factors of G3 Pancreatic Neuroendocrine Carcinomas: A Consecutive Analysis Based on Previous Study Results

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Abstract: Background: In 2017, the World Health Organization (WHO) officially defined pancreatic neuroendocrine neoplasms into well-differentiated tumors, namely G1/G2/G3 pancreatic neuroendocrine tumors, and poorly differentiated carcinomas referring to G3 pancreatic neuroendocrine carcinomas (p-NECs). However, the surgical outcomes and prognostic factors of G3 p-NECs are still unclear. **Methods:** We retrospectively collected and analyzed the data of eligible patients with G3 p-NECs defined by the WHO 2017 grading classification. **Results:** We eventually identified 120 patients with G3 p-NECs, including 72 females and 48 males, with a median age of 53 y. The 3-year overall survival (OS) of G3 p-NECs by Kaplan–Meier method was 37.3%. The 3-year OS for functional G3 p-NECs was 57.4%, which was statistically longer than 23.0% of non-functional ones ($p = 0.002$). Patients with surgical resection presented a significantly better 3-year OS than those with palliative operation (43.3% vs. 13.1%; $p < 0.001$). The 3-year OS for Stage I, Stage II, Stage III, and Stage IV was 87.1%, 56.5%, 12.9%, and not applicable, respectively ($p < 0.001$). We demonstrated in a Cox regression model that palliative operation ($p = 0.013$), vascular infiltration ($p = 0.039$), lymph node involvement ($p = 0.024$), and distant metastasis ($p = 0.016$) were independent predictors of poor outcome for patients with surgically treated G3 p-NECs. **Conclusion:** Our data in the present analysis indicated that patients with G3 p-NECs could significantly benefit from surgical resection. Meanwhile, vascular infiltration, lymph node involvement, and distant metastasis were independent predictors of poor outcome for these patients.

Keywords: pancreatic neuroendocrine carcinomas; WHO; grading; resection; prognosis

1. Introduction

Pancreatic neuroendocrine neoplasms (p-NENs), i.e., islet cell tumors, are a group of highly heterogeneous tumors with significantly different clinical features [1–5]. P-NENs comprise about 1% to 2% of all clinically detected pancreatic tumors, with an estimated annual worldwide incidence of 0.25 to 0.5 in 100,000 individuals [1,4,6]. However, p-NENs have been increasingly diagnosed during the past several decades, probably due to improvements in both clinicians' awareness of this disease and the ability to detect localized and asymptomatic tumors by imaging modalities [1,6–8].

The first case of p-NENs was reported over 100 years ago [9], though we still find it difficult to classify p-NENs into prognostic groups for survival analysis due to their rarity and heterogeneity. In 2010, based on the well-known histological definitions of p-NENs in 2000 [10], the World Health Organization (WHO) classified p-NENs into Grading 1 (G1) pancreatic neuroendocrine tumors (p-NETs), G2 p-NETs, and G3 pancreatic

neuroendocrine carcinomas ("G3 p-NECs") [11], which was first introduced by the European Neuroendocrine Tumor Society (ENETS) [12]. Furthermore, in 2017, WHO officially separated p-NENs into two different groups, including well-differentiated tumors, namely G1/G2/G3 p-NETs, and poorly differentiated carcinomas referring to G3 p-NECs [13].

The WHO 2017 grading system for p-NENs aimed to improve the prediction of clinical outcomes and to help clinicians to select better therapeutic strategies for patient care and management [13]. Our previous research demonstrated that the WHO 2017 grading classification has made an important improvement on the WHO 2010 grading criteria because of its better ability to classify p-NENs into prognostic groups [14]. Nevertheless, the surgical outcomes and prognostic factors of the newly defined G3 p-NECs are still unclear. Therefore, with the results of our previous study [14], we here attempted to carry out an in-depth analysis of the clinical characteristics of G3 p-NECs. Moreover, we emphasized demonstrating the prognostic predictors for the survival of G3 p-NECs.

2. Materials and Methods

2.1. Patients Enrollment

This was a retrospective study referring to patients with G3 p-NECs undergoing surgical treatment between January 2002 and May 2020 in one of the largest medical institutes in China. We enrolled patients who were surgically treated, either by resection or biopsy, while those without any operation were excluded. With the agreement of the principles of Helsinki Declaration [15], the written informed consent of the present study was obtained on admission from all patients. Our research was approved by the Institutional Review Board and Ethics Committee of our hospital, as it was a consecutive analysis based on previous study results [14]. As before [14,16–18], the present analysis was performed according to tumor site in pancreas, tumor size, histopathology, and type of operation; demographic data included sex, age, and symptoms at presentation; treatment-related factors included date and type of operation, surgical complications, length of stay in hospital, and so on.

2.2. Tumor Features

In the present study, we defined G3 p-NECs as functional if patients presented symptoms related to hormone overproduction, such as insulinoma, gastrinoma, glucagonoma, etc., and nonfunctional if they did not. According to the documented definitions [10,14,19,20], poorly differentiated tumors manifest nodular or solid architecture lack of organoid traits, usually with high nucleocytoplasm ratio and multifocal or extensive tumor necrosis. In light of the WHO 2017 grading classification for p-NENs, G3 p-NECs were defined as having >20 mitoses per 10 high power fields (HPFs) or a Ki-67 proliferation index >20%, with poorly differentiated small cell or large cell features [13] All cases were staged according to the tumor-node-metastasis (TNM) system introduced by the American Joint Committee on Cancer (AJCC) 8th staging manual [21]. For enrolled patients, all surgical specimens from tumor tissues were re-stained with hematoxylin-eosin and immunohistochemical methods, which were microscopically reviewed by experienced pathologists in our institution. The histopathological features of all p-NECs were systematically documented in the prepared tabulations, as we performed in the previous study [14].

2.3. Statistical Analyses

In the present study, we report quantitative variables as means with standard deviation (SD) or medians and categorical variables as numbers with their frequencies as proportions (%). Similar to our previous studies [14,16–18], we conducted the follow-up by telephone, e-mail, mail, or outpatient clinic review between July 2019 and February 2021. Overall survival (OS) was calculated either as the time in months between the date of surgery and the date of death or last follow-up and presented as either median survival time (MST) or OS with a hazard ratio (HR) and 95% confidence intervals (CIs). We applied the Kaplan–Meier (K–M) method to generate the OS estimates and compared them by the log-rank test.

Finally, we performed univariate and multivariate analysis in Cox regression proportional hazards model to demonstrate the prognostic predictors for the outcome of G3 p-NECs. Statistical analyses were performed using IBM SPSS 25.0 statistical software, which was defined as significant if the *p*-value was less than 0.05.

3. Results

3.1. Baseline Demographics and Tumor Characteristics

As Table 1 presents, we identified 120 eligible patients with G3 p-NECs in this research. Our study cohort was composed of 72 females and 48 males, with a mean age at diagnosis of 50.2 ± 13.3 y and a median of 53 y (ranging from 14 y to 86 y). Most patients (84.2%) were diagnosed after the year of 2010 and most cases (79.2%) were solitary. The mean tumor diameter was 6.8 ± 3.5 cm, with a median of 5 cm (ranging from 1.5 cm to 13.5 cm). There were 66 tumors detected in the body and tail of the pancreas, while 54 were in the head and uncinate. In light of patients' clinical manifestations and the tumors' functional status, 50 patients presented as functional, in which insulinomas accounted for the majority (36 cases). As for 70 patients with nonfunctional G3 p-NECs, abdominal pain and distension was the main clinical manifestation of 46 patients, while abdominal mass and weight loss was that of 38 patients, with jaundice being that of 25 patients. Meanwhile, there were 37 patients with incidental diagnosis who might be detected by routine physical examinations.

Abdominal US, CT, and MRI were, respectively, performed in 94, 68, and 72 patients, whose positivity rate was 74.5%, 85.3%, and 83.3%. A total of 75 patients received postoperative medical therapy, including 24 cases with molecular targeting treatment and 51 with traditional platinum-based chemotherapy. The median Ki-67 positive index and mitotic rate of G3 p-NECs was respectively 62% (ranging from 23% to 90%) and 40 per 10 HPFs (ranging from 28 per 10 HPFs to 62 per 10 HPFs). For the functional group, the Ki-67 positive index ranged from 23% to 75%, with a median of 46%, while that of the nonfunctional ones ranged from 31% to 90%, with a median of 71%. In terms of the TNM staging system, there were 25 patients presenting with vascular infiltration, 55 cases with lymph node involvement, and 33 with distant metastasis, leading to a distribution of 22, 35, 30, and 33 patients, respectively, in Stage I, Stage II, Stage III, and Stage IV.

3.2. Surgical Treatment and Postoperative Complication

As Table 2 presents, surgical resection was successfully performed for 94 patients, while a palliative operation was carried out for 26 patients. For patients with resections, 74 cases were of R0 status with both grossly and microscopically negative surgical margin, while 20 patients showed either grossly or microscopically positive surgical margin (i.e., R1/R2). Referring to the detailed surgical procedure, distal pancreatectomy (32.5%) and pancreaticoduodenectomy (30.8%) were the two most common approaches, followed by local resection of pancreatic tumor (referring to enucleation; 8.3%). A biopsy was performed for all patients with palliative operation (21.7%) in order to acquire the enough surgical specimens from tumor tissues to confirm the diagnosis of G3 p-NECs. The anesthesia grade from I to V by the American Society of Anesthesiologists was respectively evaluated in 14, 34, 45, 27, and 0 patients. There were 36 patients who experienced perioperative blood transfusion, with a mean volume of 420.5 ± 118.8 mL and a median of 400 mL (ranging from 100 mL to 1500 mL). The mean duration of operation was 202.4 ± 82.5 min, with a median of 180 min (ranging from 80 min to 510 min). A total of 42 patients had intensive care unit (ICU) in-hospital stays postoperatively, with a mean duration of 4.2 ± 1.8 d and a median of 3 d (ranging from 1 d to 10 d). The mean duration of postoperative and total in-hospital stay was, respectively, 12.4 ± 8.6 d and 21.2 ± 14.4 d, with a separate median of 9 d (ranging from 3 d to 36 d) and 11 d (ranging from 7 d to 52 d).

Table 1. Clinical characteristics of G3 p-NECs in the present study (N = 120).

Factor	Patients	
	No.	**%**
Patient sex		
Female	72	60.0
Male	48	40.0
Patient age at diagnosis, y		
Mean	50.2 ± 13.3	
Median (Range)	53 (14–86)	
Patient diagnostic period		
Before 2010	19	15.8
After 2010	101	84.2
Tumor number		
Solitary	95	79.2
Multiple	25	20.8
Tumor diameter, cm		
Mean	6.8 ± 3.5	
Median (Range)	5 (1.5–13.5)	
Tumor site		
Head and uncinate	54	45.0
Body and tail	66	55.0
Tumor functional status		
Functional tumors	50	41.7
Insulinoma	36	30.0
Others	14	11.7
Nonfunctional tumors	70	58.3
Abdominal pain and distension	46	38.3
Abdominal mass and weight loss	38	31.7
Jaundice	25	20.8
Incidental diagnosis	37	30.8
Preoperative imaging examinations		
US positive (N = 94)	70	74.5
CT positive (N = 68)	58	85.3
MRI positive (N = 72)	60	83.3
Postoperative medical therapy	75	62.5
Molecular targeting treatment	24	20.0
Traditional platinum-based chemotherapy	51	42.5
Ki-67 index, (%)		
Mean	55	
Median (Range)	62 (23–90)	
Mitotic rate, (per 10HPFs)		
Mean	38	
Median (Range)	40 (28–62)	
Vascular infiltration	25	20.8
Lymph node involvement	55	45.8
Distant metastasis	33	27.5
Tumor TNM staging system		
Stage I	22	18.3
Stage II	35	29.2
Stage III	30	25.0
Stage IV	33	27.5
Patient prognosis		
Follow-up time, mons		
Mean	48.8 ± 15.6	
Median (Range)	56.8 (10.3–176.4)	
Out of contact	20	16.7
Dead at follow-up	55	55.0
Estimated 3-year OS		37.30%
MST, mons.		30.6

Abbreviations: G: grading; p-NECs: pancreatic neuroendocrine carcinomas; US: ultrasound; CT; computed tomography; MRI: magnetic resonance imaging; HPFs: high power fields; TNM: tumor-node-metastasis; OS: overall survival; MST: median survival time.

Table 2. Surgical treatment and postoperative complication of G3 p-NECs in the present study (N = 120).

Factor	Patients	
	No.	**%**
Operation classification		
Surgical resection	94	78.3
Palliative operation	26	21.7
Surgical margin (N = 94)		
R0	74	78.7
R1/R2	20	21.3
Surgical procedure		
Local resection of pancreatic tumor	10	8.3
Distal pancreatectomy	39	32.5
Pancreaticoduodenectomy	37	30.8
Biopsy	26	21.7
Others	8	6.7
Anesthesia grade by ASA		
I	14	11.7
II	34	28.3
III	45	37.5
IV	27	22.5
V	0	0
Volume of perioperative blood transfusion, ml	36	30.0
Mean	420.5 ± 118.8	
Median (Range)	400 (100–1500)	
Duration of operation, min		
Mean	202.4 ± 82.5	
Median (Range)	180 (80–510)	
Duration of ICU in-hospital stay, d	42	35.0
Mean	4.2 ± 1.8	
Median (Range)	3 (1–10)	
Duration of postoperative in-hospital stay, d		
Mean	12.4 ± 8.6	
Median (Range)	9 (3–36)	
Duration of total in-hospital stay, d		
Mean	21.2 ± 14.4	
Median (Range)	11 (7–52)	
Total in-hospital cost, RMB		
Mean	50,212.4 ± 21,208.6	
Median (Range)	56,450 (28,905–10,983)	
Postoperative complication	30	25.0
Pancreatic fistula	21	17.5
Intra-abdominal infection	10	8.3
Pulmonary infection	9	7.5
Wound infection	5	4.2
Delayed gastric emptying	5	4.2
Intestinal obstruction	4	3.3
Intra-abdominal hemorrhage	3	2.5
Biliary fistula	2	1.7
Intestinal fistula	2	1.7
In-hospital death	1	0.8
Reoperation	5	4.2
Wound infection	2	1.7
Pancreatic fistula	1	0.8
Intra-abdominal hemorrhage	1	0.8
Intra-abdominal infection	1	0.8

Abbreviations: G: grading; p-NECs: pancreatic neuroendocrine carcinomas; R: radical; ASA: American Society of Anesthesiologists; ICU: intensive care unit; RMB: renminbi.

Of all the surgically treated patients with G3 p-NECs, 30 experienced postoperative complications, with a morbidity of 25.0% (Table 2). Pancreatic fistulas occurred in 21 patients, which was the most common postoperative complication (17.5%), followed by intra-abdominal infection (8.3%) and pulmonary infection (7.5%). Other complications, such as wound infection (4.2%), delayed gastric emptying (4.2%), intestinal obstruction (3.3%), intra-abdominal hemorrhage (2.5%), biliary fistula (1.7%), and intestinal fistula (1.7%), were uncommon. There was 1 in-hospital death caused by intra-abdominal hemorrhage, with a mortality of 0.8%. A total of 5 patients experienced reoperation (4.2%), including 2 cases for wound infection, 1 for pancreatic fistula, 1 for intra-abdominal hemorrhage, and 1 for intra-abdominal infection. All other postoperative complications could be treated well through non-operational therapies, such as appropriate medical treatments and unobstructed drainages.

3.3. Survival Estimates and Prognostic Analyses

The mean follow-up time of 100 patients was 48.8 ± 15.6 months, with a median of 56.8 months (ranging from 10.3 months to 176.4 months), while 20 patients were out of contact (16.7%). When the follow-up ended, there were 45 patients alive, whereas 55 were dead due to the progression of disease (55.0%). According to the K–M method, the accumulative 3-year OS of the entire cohort was 37.3%, with a MST of 30.6 months (95% CIs: 24.8–36.3; Figure 1). The 3-year OS and MST of functional G3 p-NECs were respectively 57.4% and 42.3 months (95% CIs: 30.5–54.1), while those of nonfunctional ones were 23.0% and 25.3 months (95% CIs: 20.8–29.7; $p = 0.002$; Figure 2). Patients with surgical resection obtained a 3-year OS of 43.4% and a MST of 34.5 months (95% CIs: 29.7–39.2), which was statistically better than that of patients with palliative operation (13.1%; 14.3 mons (95% CIs: 11.9–16.7); $p < 0.001$; Figure 3). The OS at 3 years for patients in Stage I, Stage II, Stage III, and Stage IV was, respectively, 87.1%, 56.5%, 12.9%, and not applicable, with a MST of 55.4 months (95% CIs: 45.3–65.4), 41.2 months (95% CIs: 34.6–47.7), 26.8 months (95% CIs: 23.6–29.9), and 14.8 months (95% CIs: 11.7–17.8). To be specific, survivals of patients in Stage I or Stage II were statistically better than those in Stage III ($p < 0.001$, $p = 0.008$, respectively) or Stage IV ($p < 0.001$, $p < 0.001$, respectively; Figure 4). Meanwhile, survival differences when comparing Stage I with Stage II or Stage III with Stage IV were also significant ($p = 0.011$, $p = 0.001$, respectively; Figure 4).
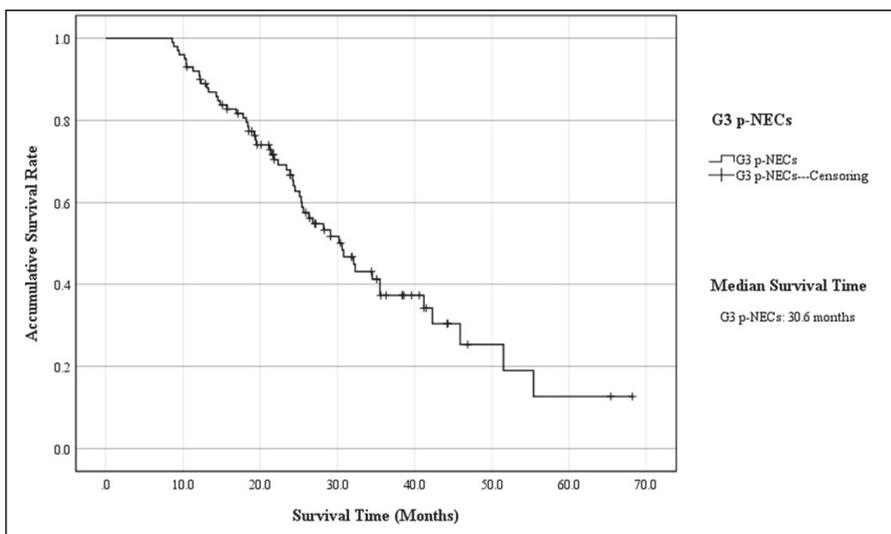
Figure 1. Kaplan–Meier estimates for the OS of G3 p-NECs.
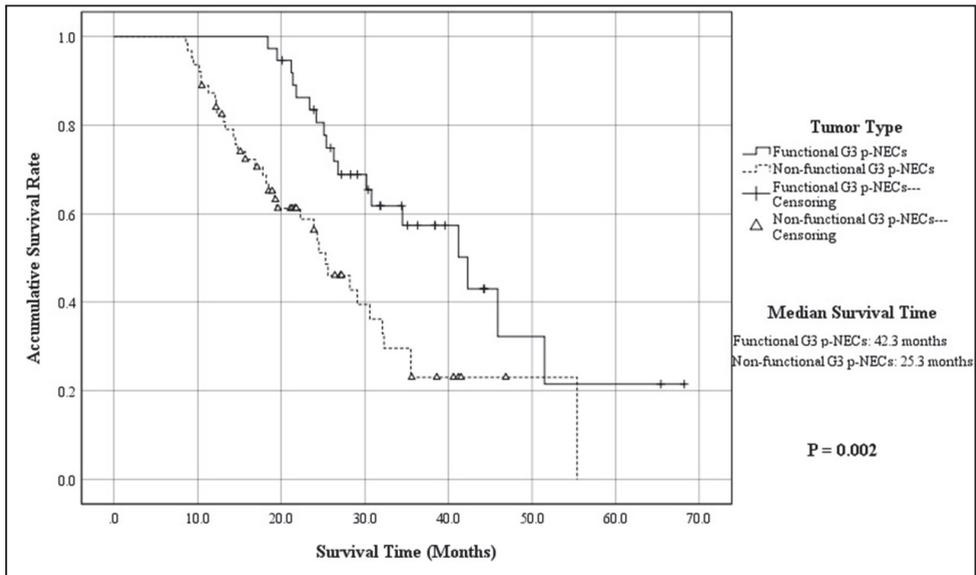
Figure 2. Kaplan–Meier estimates for the OS of G3 p-NECs, according to the tumor type.
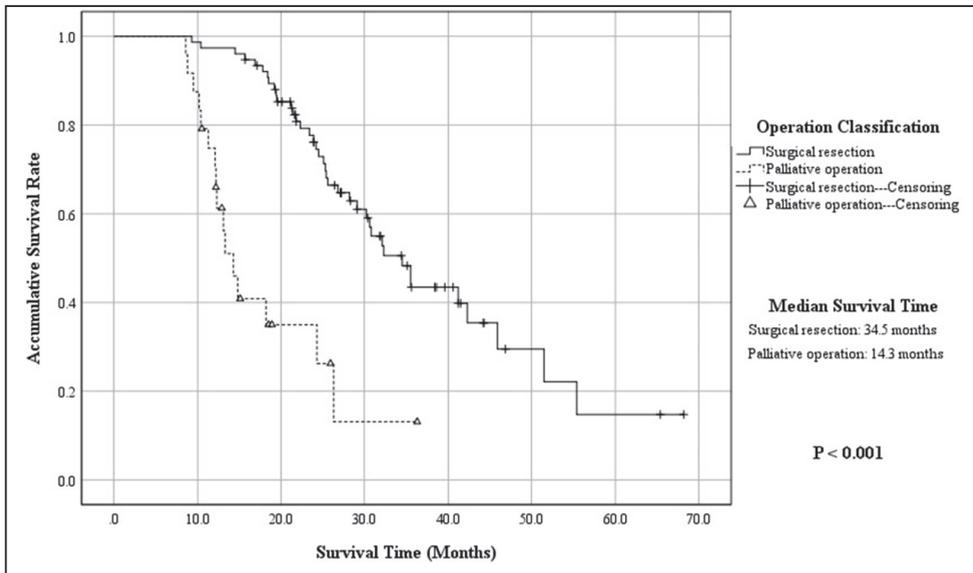
Figure 3. Kaplan–Meier estimates for the OS of G3 p-NECs, according to the operation classification.

As Table 3 listed, sex, age, tumor site, incidental diagnosis, duration of operation, duration of postoperative in-hospital stay, ICU in-hospital stay, perioperative blood transfusion, and postoperative complication presented no notable differences in univariate analyses ($p > 0.05$). According to the subsequent multivariate analyses, tumor type, tumor diameter, anesthesia grade, surgical margin, and postoperative medical therapy were not notably significant ($p > 0.05$), while operation classification ($p = 0.013$), vascular infiltration ($p = 0.039$), lymph node involvement ($p = 0.024$), and distant metastasis ($p = 0.016$) were independent predictors for the prognosis of G3 p-NECs.
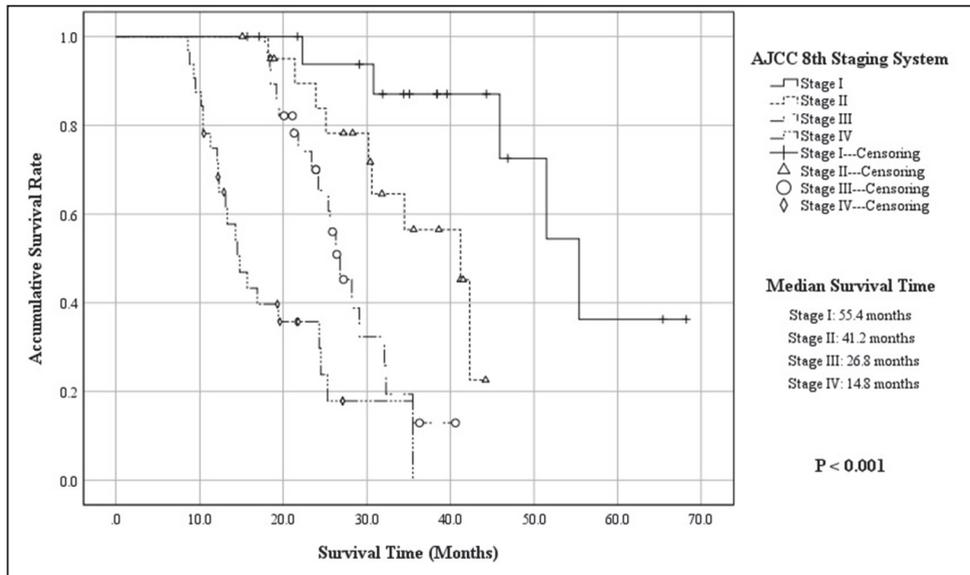
Figure 4. Kaplan–Meier estimates for the OS of G3 p-NECs, according to the AJCC 8th staging system.

Table 3. Univariate and multivariate analyses of factors influencing the prognosis of G3 p-NECs in the present study (N = 120).

Factor	Univariate Analysis		Multivariate Analysis	
	HR (95% CIs)	*p*	HR (95% CIs)	*p*
Sex				
Male [A]				
Female	0.894 (0.554–2.113)	0.625		
Age, y				
<Median				
≥Median	1.541 (0.509–2.639)	0.091		
Tumor site				
Head and uncinate				
Body and tail	1.083 (0.516–1.522)	0.493		
Tumor type				
Functional				
Nonfunctional	1.725 (0.652–3.356)	**0.031**	0.914 (0.673–1.487)	0.619
Incidental diagnosis				
No				
Yes	1.003 (0.357–1.766)	0.213		
Tumor diameter				
<Median				
≥Median	1.863 (0.387–2.263)	**0.047**	0.557 (0.267–1.013)	0.652
Anesthesia grade				
I/II				
III/IV/V	1.554 (0.446–2.731)	**0.038**	0.791 (0.381–1.451)	0.443
Operation classification				
Resection				
Palliative	3.215 (0.379–8.236)	**<0.001**	1.493 (0.513–4.343)	0.013

Table 3. *Cont.*

Factor	Univariate Analysis		Multivariate Analysis	
	HR (95% CIs)	*p*	HR (95% CIs)	*p*
Surgical margin				
R0				
R1/R2	1.813 (0.425–2.091)	**0.012**	1.113 (0.453–1.853)	0.092
Duration of operation				
<Median				
≥Median	1.345 (0.521–2.892)	0.113		
Duration of postoperative in-hospital stay				
<Median				
≥Median	1.115 (0.371–1.983)	0.305		
Perioperative blood transfusion				
No				
Yes	1.563 (0.476–2.093)	0.235		
ICU in-hospital stay				
No				
Yes	1.212 (0.674–1.814)	0.354		
Postoperative complication				
No				
Yes	1.315 (0.784–2.336)	0.549		
Postoperative medical therapy				
TPC				
MTT	1.925 (0.486–3.065)	**0.037**	1.094 (0.334–1.985)	0.184
Vascular infiltration				
No				
Yes	2.412 (0.731–6.126)	**<0.001**	5.232 (1.263–11.225)	**0.039**
Lymph node involvement				
No				
Yes	3.335 (0.982–8.426)	**0.029**	1.903 (0.329–5.013)	**0.024**
Distant metastasis				
No				
Yes	4.576 (0.775–12.435)	<0.001	2.493 (0.416–13.436)	**0.016**

[A]: The above related factor was regarded as a reference in Cox analysis. Abbreviation: G: grading; p-NECs: pancreatic neuroendocrine carcinomas; HR: hazard ratio; CIs: confidence intervals; R: radical; ICU: intensive care unit; TPC: traditional platinum-based chemotherapy; MTT: molecular targeting treatment.

4. Discussion

P-NENs are a heterogeneous group of malignancies [1–3]. The grading classification based on mitotic counts and Ki-67 proliferation index by WHO in 2010 [11] has reflected great clinical value with widespread acceptance [22–25]. However, accumulated studies have demonstrated that those "G3 p-NECs" by the WHO 2010 grading system were morphologically and biologically heterogeneous, with different clinical-pathological features and long-term survivals [26–30]. Therefore, as reviewed by Julie et al. in their report [20], the heterogeneity of "G3 p-NECs" has promoted the emergence of the new WHO grading classification in 2017 [13], whose clinical value has just been validated by our studying team [14].

The present research was a consecutive analysis based on our previous report [14], because as a new sub-category of p-NENs, the surgical outcomes and prognostic factors of G3 p-NECs have not been comprehensively analyzed before. As reported [20,27,31], the clinical features of G3 p-NECs were very similar to typical pancreatic exocrine adenocarcinomas

(p-EACs). Our analyses revealed that patient sex of G3 p-NECs had a slight female predominance (60%) with a median age of 53 y and that G3 p-NECs more frequently involved the body and tail of the pancreas (55%). These findings were basically in agreement with what was reported in our previous study [14]. Meanwhile, nonfunctional tumors accounted for most G3 p-NECs (70%), in which abdominal pain and distension, abdominal mass and weight loss, and jaundice were the main clinical presentations (38.3%, 31.7%, and 20.8% respectively), while incidental diagnosis was also obtained by physical examinations or others from 30.8% patients. Sorbye et al. reported that obstructive jaundice or nonspecific abdominal complaints might be the only signs or symptoms available to the suspicion of G3 p-NECs [32]. We here revealed that functional G3 p-NECs obtained a notably better survival than nonfunctional ones (57.4% vs. 23.0%; $p = 0.002$; Figure 2), probably due to earlier diagnosis based on clinical symptoms. However, tumor type still could not be a significant predictor for the prognosis of G3 p-NECs in the Cox regression model ($p = 0.061$; Table 3), as we have demonstrated [17].

Most G3 p-NECs were very mitotically active and cases with >40 to 50 mitoses per HPFs or Ki-67 proliferation index >50% were frequently observed [26–29]. Similarly, the median Ki-67 index and mitotic rate of the entire group were respectively 62% and 40 per 10 HPFs. As for clinical stage of G3 p-NECs, we previously demonstrated that the AJCC 8th TNM staging system originally applied to p-EACs was applicable for G3 p-NECs due to its better prognostic stratification and more accurate predicting ability [17]. According to our present analyses, we also succeeded in classifying G3 p-NECs into 4 groups with significantly different survivals by this staging system ($p < 0.001$; Figure 4).

G3 p-NECs could be treated by both surgical and medical therapy according to their clinical features, especially tumor grade and clinical stage [3–5,26–29]. In our present study, surgical resection was carried out for 94 patients with G3 p-NECs, in which distal pancreatectomy and pancreaticoduodenectomy were the two main procedures (32.5% and 30.8%; respectively), while palliative operation with biopsy was performed for 21.7% cases. As we proved in Table 3, operation classification was an independent predictor for the prognosis of G3 p-NECs ($p = 0.013$), in which patients could significantly benefit from surgical resection more than palliative operation (43.4% vs. 13.1%; $p < 0.001$; Figure 3). Moreover, patients with R0 surgical margin showed longer survival compared with those with R1/R2 margin ($p = 0.012$), while the surgical margin still failed to be proven as an independent predictor in the multivariate analyses ($p = 0.092$). Interestingly, we here had 10 cases of G3 p-NECs in which local resections of pancreatic tumor (referring to enucleation) were performed. We currently agree that a more radical approach for G3 p-NECs would be considered standard (identically to p-EACs). However, G3 p-NECs in this research were finally diagnosed by postoperative pathological examinations from the surgical specimens, which meant we did not know the neuroendocrine phenotype of the pancreatic lesion during operation. Moreover, enucleation of pancreatic tumor was carried out mainly in the early years when the biological behaviors of G3 p-NECs were not clear. It would be interesting to know the prognostic difference among distal pancreatic resection and pancreaticoduodenectomy with local resection. However, the power of this analysis would indeed be insufficient, due to the small number of cases with enucleation (only 10 cases).

When the diagnosis of either "G3 p-NECs" by WHO 2010 grading classification or the present G3 p-NECs by WHO 2017 grading criteria was made by the postoperative pathological examinations, adjuvant therapy was routinely indicated in our hospital. However, drugs for the medical therapy varied over time, from the molecular targeted therapy at the beginning, such as sunitinib, everolimus, and octreotide, to the platinum-based drugs proposed by guidelines later [33], such as cisplatin and oxaliplatin. In the present study, we identified 75 patients who received postoperative medical therapy. Due to the small number of cases with each drug, we classified these patients into 24 cases with molecular targeting treatment and 51 with platinum-based chemotherapy. We found that patients could benefit from platinum-based chemotherapy, presenting a statistically longer survival than those

with molecular targeting treatment ($p = 0.037$; Table 3), but postoperative medical therapy could not be a significant predictor for the outcome of G3 p-NECs ($p = 0.184$; Table 3), as we reported before [17].

Our study also had some limitations [14]. First, it was a retrospective study. Secondly, the accumulative OS was estimated by K–M methods. Then, our analysis derived from one single medical institution. Finally, we only enrolled patients who were surgically treated, either by resection or biopsy, while those without any operation were excluded. Therefore, a particular implication for G3 p-NECs, particularly those with metastatic disease at presentation might be unsuitable for any operation, given that surgery would not be considered as standard management for some patients. Moreover, surgery might not be strongly recommended from this case series since the better outcomes could be mainly related to lead time bias. With the above limitations, our present study still achieved the expected goal and will be of great value in guiding the treatment and prognosis of G3 p-NECs.

5. Conclusions

In sum, based on the studying results of our previous research, we carried out a consecutive analysis on the surgical outcomes and prognostic factors of G3 p-NECs in the present study. According to our demonstrations, G3 p-NECs could notably benefit from surgical resection, while vascular infiltration, lymph node involvement, and distant metastasis were independent predictors of poor prognosis for these patients.

References

1. Yao, J.C.; Hassan, M.; Phan, A.; Dagohoy, C.; Leary, C.; Mares, J.E.; Abdalla, E.K.; Fleming, J.B.; Vauthey, J.N.; Rashid, A.; et al. One hundred years after "carcinoid": Epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J. Clin. Oncol.* **2008**, *26*, 3063–3072. [CrossRef]
2. Asa, S.L. Pancreatic endocrine tumors. *Mod. Pathol.* **2011**, *24* (Suppl. 2), S66–S77. [CrossRef]
3. McKenna, L.R.; Edil, B.H. Update on pancreatic neuroendocrine tumors. *Gland Surg.* **2014**, *3*, 258–275. [PubMed]
4. Turaga, K.K.; Kvols, L.K. Recent progress in the understanding, diagnosis, and treatment of gastroenteropancreatic neuroendocrine tumors. *CA Cancer J. Clin.* **2011**, *61*, 113–132. [CrossRef] [PubMed]
5. Kloppel, G. Classification and pathology of gastroenteropancreatic neuroendocrine neoplasms. *Endocr. Relat. Cancer* **2011**, *18* (Suppl. 1), S1–S16. [CrossRef]
6. Hallet, J.; Law, C.H.L.; Cukier, M.; Saskin, R.; Liu, N.; Singh, S. Exploring the rising incidence of neuroendocrine tumors: A population-based analysis of epidemiology, metastatic presentation, and outcomes. *Cancer* **2015**, *121*, 589–597. [CrossRef] [PubMed]

7.   Krampitz, G.W.; Norton, J.A. Pancreatic neuroendocrine tumors. *Curr. Probl. Surg.* **2013**, *50*, 509–545. [CrossRef]
8.   Keutgen, X.M.; Nilubol, N.; Kebebew, E. Malignant functioning neuroendocrine tumors of the pancreas: A survival analysis. *Surgery* **2016**, *159*, 1382–1389. [CrossRef]
9.   Nichholls, A.G. Simple adenoma of the pancreas arising from an island of Langerhans. *J. Med. Res.* **1902**, *8*, 385–395.
10. Solcia, E.; Kloppel, G.; Sobin, L. Histological Typing of Endocrine Tumours. In *World Health Organization International Histological Classification of Tumours*, 2nd ed.; Springer: Berlin, Germany, 2000.
11. Rindi, G.; Arnold, R.; Bosman, F.T. Nomenclature and classification of neuroendocrine neoplasms of the digestive system. In *WHO Classification of Tumours of the Digestive System*, 4th ed.; Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D., Eds.; International Agency for Research on Cancer: Lyon, France, 2010; pp. 13–40.
12. Rindi, G.; Klöppel, G.; Alhman, H.; Caplin, M.; Couvelard, A.; de Herder, W.W.; Erikssson, B.; Falchetti, A.; Falconi, M.; Komminoth, P.; et al. TNM staging of foregut (neuro)endocrine tumors: A consensus proposal including a grading system. *Virchows Arch.* **2006**, *449*, 395–401. [CrossRef]
13. Klöppel, G.; Couvelard, A.; Hruban, R.H. Tumours of the endocrine pancreas. In *WHO Classification of the Tumours of Endocrine Organs*, 4th ed.; Lloyd, R.V., Osamura, R.Y., Klöppel, G., Rosai, J., Eds.; International Agency for Research on Cancer: Lyon, France, 2017; pp. 175–207.
14. Yang, M.; Zeng, L.; Ke, N.-W.; Tan, C.-L.; Tian, B.-L.; Liu, X.-B.; Xiang, B.; Zhang, Y. World Health Organization grading classification for pancreatic neuroendocrine neoplasms: A comprehensive analysis from a large Chinese institution. *BMC Cancer* **2020**, *20*, 906. [CrossRef] [PubMed]
15. Crawley, F.P. The Limits of the Declaration of Helsinki. In *Address to Scientific Session*; World Medical Association General Assembly: Helsinki, Finland, 2012.
16. Yang, M.; Zhang, Y.; Zeng, L.; Ke, N.W.; Tan, C.L.; Xiang, B.; Liu, X.B. Prognostic Validity of the American Joint Committee on Cancer Eighth Edition TNM Staging System for Surgically Treated and Well-Differentiated Pancreatic Neuroendocrine Tumors: A Comprehensive Analysis of 254 Consecutive Patients From a Large Chinese Institution. *Pancreas* **2019**, *48*, 613–621.
17. Yang, M.; Zhang, Y.; Zeng, L.; Ke, N.-W.; Tan, C.-L.; Tian, B.-L.; Xiang, B.; Liu, X.-B. Survivals of patients with surgically treated and High-grade pancreatic neuroendocrine carcinomas: A comparative study between two American Joint Committee on Cancer 8th tumor-node-metastasis staging systems. *Eur. J. Surg. Oncol.* **2019**, *45*, 1054–1060. [CrossRef]
18. Deng, B.Y.; Yang, M.; Wen, J.Y.; Hou, S.Z.; Chen, Y.; Tian, B.L.; Liu, X.B.; Zhang, Y. Survivals of patients with pancreatic neuroendocrine carcinomas: An in-depth analysis by the American Joint Committee on Cancer 8th tumor-node-metastasis staging manual. *Medicine* **2020**, *99*, e18736. [CrossRef] [PubMed]
19. Solcia, E.; Capella, C. Endocrine tumours of the gastrointestinal tract. Histologic Typing of Endocrine Tumours. In *WHO International Histological Classification of Tumours*; Springer: New York, NY, USA, 2000; pp. 57–67.
20. Julie, G.M.; Nosé, V. Neoplasms of the Neuroendocrine Pancreas: An Update in the Classification, Definition, and Molecular Genetic Advances. *Adv. Anat. Pathol.* **2019**, *26*, 13–30.
21. Kakar, S.; Pawlik, T.M.; Allen, P.J. (Eds.) *AJCC Cancer Staging Manual*, 8th ed.; Springer: New York, NY, USA, 2017.
22. Dolcetta-Capuzzo, A.; Villa, V.; Albarello, L.; Franchi, G.M.; Gemma, M.; Scavini, M.; Di Palo, S.; Orsenigo, E.; Bosi, E.; Doglioni, C.; et al. Gastroenteric neuroendocrine neoplasms classification: Comparison of prognostic models. *Cancer* **2013**, *119*, 36–44. [CrossRef] [PubMed]
23. Kunz, P.L.; Reidy-Lagunes, D.; Anthony, L.B.; Bertino, E.M.; Brendtro, K.; Chan, J.A.; Chen, H.; Jensen, R.T.; Kim, M.K.; Klimstra, D.S.; et al. Consensus Guidelines for the Management and Treatment of Neuroendocrine Tumors. *Pancreas* **2013**, *42*, 557–577. [CrossRef]
24. Yang, M.; Tian, B.-L.; Zhang, Y.; Su, A.-P.; Yue, P.-J.; Xu, S.; Wang, L. Evaluation of the World Health Organization 2010 Grading System in Surgical Outcome and Prognosis of Pancreatic Neuroendocrine Tumors. *Pancreas* **2014**, *43*, 1003–1008. [CrossRef]
25. Yang, M.; Ke, N.-W.; Zhang, Y.; Zeng, L.; Tan, C.-L.; Zhang, H.; Mai, G.; Tian, B.-L.; Liu, X.-B. Survival Analyses for Patients With Surgically Resected Pancreatic Neuroendocrine Tumors by World Health Organization 2010 Grading Classifications and American Joint Committee on Cancer 2010 Staging Systems. *Medicine* **2015**, *94*, e2156. [CrossRef]
26. Sorbye, H.; Welin, S.; Langer, S.W.; Vestermark, L.W.; Holt, N.; Osterlund, P.; Dueland, S.; Hofsli, E.; Guren, M.G.; Ohrling, K.; et al. Predictive and prognostic factors for treatment and survival in 305 patients with advanced gastrointestinal neuroendocrine carcinoma (WHO G3): The NORDIC NEC study. *Ann. Oncol.* **2013**, *24*, 152–160. [CrossRef]
27. Basturk, O.; Yang, Z.; Tang, L.H.; Hruban, R.H.; Adsay, V.; McCall, C.; Krasinskas, A.M.; Jang, K.-T.; Frankel, W.L.; Balci, S.; et al. The high-grade (WHO G3) pancreatic neuroendocrine tumor category is morphologically and biologically heterogenous and includes both well differentiated and poorly differentiated neoplasms. *Am. J. Surg. Pathol.* **2015**, *39*, 683–690. [CrossRef] [PubMed]
28. Tang, L.H.; Untch, B.R.; Reidy, D.L.; O'Reilly, E.M.; Dhall, D.; Jih, L.; Basturk, O.; Allen, P.J.; Klimstra, D.S. Well-differentiated neuroendocrine tumors with a morphologically apparent high grade component: A pathway distinct from poorly differentiated neuroendocrine carcinomas. *Clin. Cancer Res.* **2016**, *22*, 1011–1017. [CrossRef] [PubMed]
29. Tang, L.H.; Basturk, O.; Sue, J.J.; Klimstra, D.S. A practical approach to the classification of WHO grade 3 (G3) well differentiated neuroendocrine tumor (WD-NET) and poorly differentiated neuroendocrine carcinoma (PD-NEC) of the pancreas. *Am. J. Surg. Pathol.* **2016**, *40*, 1192–1202. [CrossRef] [PubMed]

30. Kim, J.Y.; Hong, S.M.; Ro, J.Y. Recent updates on grading and classification of neuroendocrine tumors. *Ann. Diagn. Pathol.* **2017**, *29*, 11–16. [CrossRef]
31. Ilett, E.E.; Langer, S.W.; Olsen, I.H.; Federspiel, B.; Kjaer, A.; Knigge, U. Neuroendocrine Carcinomas of the Gastroenteropancreatic System: A Comprehensive Review. *Diagnostics* **2015**, *5*, 119–176. [CrossRef]
32. Sorbye, H.; Strosberg, J.; Baudin, E.; Klimstra, D.S.; Yao, J.C. Gastroenteropancreatic high-grade neuroendocrine carcinoma. *Cancer* **2014**, *120*, 2814–2823. [CrossRef]
33. Garcia-Carbonero, R.; Sorbye, H.; Baudin, E.; Raymond, E.; Wiedenmann, B.; Niederle, B.; Sedlackova, E.; Toumpanakis, C.; Anlauf, M.; Cwikla, J.M.; et al. ENETS consensus guidelines for high-grade gastroenteropancreatic neuroendocrine tumors and neuroendocrine carcinomas. *Neuroendocrinology* **2016**, *103*, 186–194. [CrossRef]

Article

Surgical Outcomes, Long-Term Survivals and Staging Systems of World Health Organization G3 Pancreatic Neuroendocrine Tumors

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Abstract: Background: In 2017, the World Health Organization (WHO) defined a new category of pancreatic neuroendocrine neoplasms named G3 pancreatic neuroendocrine tumors (p-NETs), whose surgical outcomes, long-term survivals and staging systems have not been well documented. Methods: Data from eligible patients with G3 p-NETs defined using the WHO 2017 grading classification at our institute were retrospectively analyzed. Results: Our study enrolled 80 patients with WHO G3 p-NETs, including 50 women and 30 men. The accumulative 5-year overall survival (OS) of G3 p-NETs was 29.7%. The current staging system by the American Joint Committee on Cancer (AJCC) failed to discriminate the survival difference between Stage II and Stage III ($p = 0.172$), while notable differences with regard to the OS were statistically offered between each stage using the modified tumor–node–metastasis (mTNM) staging system (all $p < 0.05$). The OS of patients receiving surgical resection was significantly better than those with palliative operation ($p < 0.05$). Both the current AJCC system and proposed mTNM system were independent predictors for the OS of G3 p-NETs ($p = 0.017$ and $p = 0.032$, respectively). The 95% confidence intervals of the proposed mTNM staging system were smaller than that of the current AJCC system (0.626–8.217 and 0.329–10.013, respectively), indicating a relatively more accurate predictive ability. Conclusion: Our demonstration revealed that surgical resection was an independent predictor for the favorable prognosis of patients with G3 p-NETs. Moreover, the new mTNM staging system was more suitable and practical than the current AJCC system for stratifying G3 p-NETs into prognostic groups.

Keywords: pancreatic neuroendocrine tumors; G3; resection; stage; prognosis

1. Introduction

Pancreatic neuroendocrine neoplasms (p-NENs) are a group of rare and highly heterogeneous tumors [1,2]. Although p-NENs were first reported in 1902 [3], the history of classifying patients into prognostic groups has experienced a long and complicated evolution, probably due to their rarity and heterogeneity [4].

In 2000, referring to some well-known clinic-pathological features, the World Health Organization (WHO) firstly classified p-NENs into well-differentiated endocrine tumor, well-differentiated endocrine carcinoma and poorly-differentiated endocrine carcinoma [5]. In 2006, based on the mitotic rate per 10 high power fields (HPFs) and Ki-67 proliferative index, the European Neuroendocrine Tumor Society (ENETS) specifically proposed a grading classification for p-NENs, which mainly consist of G1/G2 pancreatic neuroendocrine tumors (p-NETs) and G3 pancreatic neuroendocrine carcinomas (G3 p-NECs) [6]. Obtaining widespread acceptance in clinical practice, this ENETS system for p-NENs was officially adopted in 2010 by the WHO [7]. However, tumor differentiation based on morphology was not considered in the ENETS 2006 or the WHO 2010 grading classification, in which

morphologically well-differentiated p-NETs might have proliferative rates that met the threshold for G3 NECs [8]. Several studies have reported that G3 NECs were also heterogeneous, which included a subgroup with clinical features close to G1/G2 p-NETs on the basis of immunostaining and gene analysis results [9–13]. In 2017, referring to the features of both morphological differentiation and grading upon proliferation rate, the WHO divided p-NENs into G1/G2/G3 p-NETs and G3 p-NECs [14]. In this newly updated WHO 2017 grading system, G3 p-NETs were explicitly defined as high-grade neoplasms (Ki-67 > 20%) with a well-differentiated morphology, which have not yet been well documented in terms of their surgical outcomes, long-term survivals and staging systems.

In 2017, the 8th edition staging manual by the American Joint Committee on Cancer (AJCC) first highlighted that G1/G2 p-NETs should be staged by the ENETS tumor–node–metastasis (TNM) system primarily proposed for p-NENs [6], while G3 p-NECs be staged separately by the contemporary system originally applied to pancreatic exocrine adenocarcinomas (p-EACs) [15]. Although the AJCC 8th staging manual has made an important step towards adopting uniform systems to stratify different grading p-NENs, it has lost sight of the heterogeneous features of G3 p-NECs, as we mentioned above [9–13]. Our previous studies identified two subgroups of G3 p-NECs with varied morphological differentiations, staging features and long-term survivals [13,16]. Meanwhile, studies have demonstrated that the current AJCC system for p-NETs failed to significantly distinguish survivals between Stage I and Stage II or between Stage II and Stage III [17–20]. Recently, Zhang et al., introduced a modified tumor–node–metastasis (mTNM) system for p-NETs [21], which adopted their previously proposed nodal classification for N definitions [22], but retained the current AJCC T and M definitions (Supplementary Materials Table S1). This new mTNM system was proven to be superior to the current AJCC system and was highly appraised by specialists [23,24], for it offered statistically significant survival rates between each stage for G1/G2 p-NETs. Nevertheless, whether this proposed mTNM staging system is practical and prognostic for G3 p-NETs remained unknown.

In this research, we comprehensively assessed the clinical features, surgical outcomes, long-term survivals and prognostic factors of G3 p-NETs. Moreover, we attempted to validate and compare the clinical applications of this new mTNM staging system and the current AJCC system to G3 p-NETs.

2. Methods

Our present study was a retrospective study with regard to patients with surgically-treated and histopathologically confirmed G3 p-NETs from January 2002 to December 2020 in our hospital. Patients with a pathological diagnosis of G1/G2 p-NETs or G3 p-NECs were excluded. This study was approved by the local Institutional Review Board and Ethics Committee. In accordance with the principles of the Helsinki Declaration [25], the written informed consent was acquired on admission from all patients. The data, such as demographic baseline, clinical presentations, imaging examinations, surgical findings, pathological results and perioperative outcomes were reviewed from the patients' medical records and collected in the prepared tabulations, as in previous work [13,16,19,20].

The surgical specimens from the tumor tissues of eligible patients were re-stained with hematoxylin–eosin and immunohistochemical methods and microscopically reviewed by our experienced pancreatic pathologists according to the morphological feature, mitotic count, Ki-67 positive proliferation index, and so on. Afterwards, G3 p-NETs were defined in the light of the WHO 2017 grading classification [14]. Meanwhile, the newly proposed mTNM staging system [21] and the current AJCC system [15] were respectively applied to distribute patients into the corresponding groups.

Follow-up was mainly conducted by telephone, email, mail or outpatient clinic review between July and December of 2021, as in previous work [13,16,19,20]. The overall survival (OS) was calculated as the time in months between the date of operation and the date of death or last follow-up, which was presented as either median survival time (MST) or 5-year OS with a hazard ratio (HR) and 95% confidence intervals (CIs). Quantitative variables were

reported as median with a range, while categorical variables were presented as numbers with frequencies and proportions (%). Accumulated OS was estimated using Kaplan–Meier (K-M) methods and compared using the log-rank test. Univariate and multivariate analyses using the Cox regression proportional hazards model were performed to validate the prognostic value of potential factors for the OS of G3 p-NETs. All statistical analyses were carried out using IBM SPSS 28.0 statistical software. Differences with a two-sided *p*-value less than 0.05 were considered statistically significant.

3. Results

As shown in Table 1, the present study finally identified 80 eligible patients with G3 p-NETs. Our research consisted of 50 females and 30 males, with a median age of 50 years (ranging from 7 years to 74 years). There were 51 cases located in the body or tail of the pancreas, with a median diameter of 4.5 cm (ranging from 1.8 cm to 8.5 cm). Most G3 p-NETs were solitary (88.8%), and non-functional ones accounted for the majority in the tumor type (67.5%). There were 15 patients who were diagnosed as G3 p-NETs incidentally. In terms of the immunohistochemistry, the median Ki-67 proliferation index of G3 p-NETs was 28% (ranging from 21% to 60%), while the mitotic rate ranged from 20 per 10 HPFs to 38 per 10 HPFs, with a median of 22 per 10 HPFs. All G3 p-NETs had the presence of Chromogranin A (CgA) in the immunostaining. There were 12 cases of G3 p-NETs that showed the presence of necrosis. The median number of harvested lymph nodes was 8, ranging from 4 to 14.

Table 1. Clinical features of patients with G3 p-NETs in our study.

Factor	Patients (*n* = 80)	
	No.	**%**
Patients' gender, female	50	62.5
Patients' age at diagnosis, years	50 (7–74)	
Tumor largest diameter, cm	4.5 (1.8–8.5)	
Tumor location, pancreatic body/tail	51	63.8
Tumor number, solitary	71	88.8
Tumor type, non-functional	54	67.5
Incidental diagnosis	15	18.8
Ki-67 proliferation index, %	28 (21–60)	
Mitotic rate, per 10 HPFs	22 (20–38)	
Presence of CgA	80	100
Presence of necrosis	10	12.5
No. lymph nodes harvested (median)	8 (4–14)	
T classification by both TNM systems [A]		
T1	12	15.0
T2	14	17.5
T3	32	40.0
T4	22	27.5
Nodal metastasis (*n* = 24)		
1–3 regional lymph node metastases	15	18.8
≥4 regional lymph node metastases	9	11.3
Distant metastasis	13	16.3

Table 1. *Cont.*

Factor	Patients (*n* = 80)	
	No.	%
Current AJCC 8th staging system		
Stage I	5	6.3
Stage II	35	43.8
Stage III	27	33.8
Stage IV	13	16.1
Proposed mTNM staging system		
Stage I	9	11.3
Stage II	27	33.8
Stage III	31	38.8
Stage IV	13	16.1

Abbreviations: G: grading; p-NETs: pancreatic neuroendocrine tumors; HPFs: high power fields; CgA: Chromogranin A; TNM: tumor–node–metastasis; AJCC: American Joint Committee on Cancer; mTNM: modified tumor–node–metastasis. [A]: The definitions of T classification in the proposed mTNM staging system were the same as those in the current AJCC 8th staging system.

According to the same definitions of T status by both staging systems, there were 12, 14, 32 and 22 patients classified from T1 to T4, respectively. Nodal metastasis was detected in 24 patients, including 15 cases with 1–3 regional lymph node metastases and 9 with ≥4 regional lymph node metastases, while distant metastasis was confirmed in 13 patients. In light of the corresponding clinical stages by the current AJCC 8th system, there were respectively 5, 35, 27 and 13 patients defined as Stage I, Stage II, Stage III and Stage IV. With regard to the criteria of the proposed mTNM system, there were 9, 27, 31 and 13 patients distributed from Stage I to Stage IV, respectively.

All patients were surgically treated (Table 2), of which 62 patients received surgical resection, while 18 patients received palliative operation (such as cholangiojejunostomy, gastrojejunostomy, etc.). For patients with a resection, 56 presented both grossly and microscopically negative surgical margins. The main surgical procedures performed for G3 p-NETs were distal pancreatectomy (35.0%), pancreaticoduodenectomy (21.3%) and the local resection of pancreatic tumor (13.8%), while radical resection for selected metastatic disease was only carried out in six patients. As for the anesthesia grade by the American Society of Anesthesiologists, there were respectively 9, 23, 30, 18 and 0 patients from grade I to grade V. There were 24 patients who required perioperative blood transfusions with a median volume of 300 mL, and 15 patients who needed an intensive care unit stay with a median duration of 3 d. The median duration of operation, postoperative and total in-hospital stay was respectively 150 min, 6 d and 9 d. Postoperative complications occurred in 21 patients, with a morbidity of 26.3%, in which pancreatic fistula (12.5%), intra-abdominal infection (8.8%), delayed gastric emptying (5.0%) and intra-abdominal hemorrhage (2.5%) were the main ones. One patient underwent reoperation due to intra-abdominal hemorrhage, while all other complications were treated conservatively. There was no postoperative in-hospital death. Postoperative medical therapies were carried out for 34 patients, including 14 with novel molecular targeting treatments and 20 with traditional chemotherapies.

Table 2. Surgical outcomes and follow-up data of patients with G3 p-NETs in our study.

Factor	Patients (*n* = 80)	
	No.	**%**
Operation classification, surgical resection	62	77.5
Surgical margin (*n* = 62), radical [A]	56	90.3
Surgical procedures		
Local resection of pancreatic tumor (enucleation included)	11	13.8
Distal pancreatectomy	28	35.0
Pancreaticoduodenectomy	17	21.3
Radical resection for metastatic disease	6	7.5
Palliative operation with biopsy [B]	18	22.5
Anesthesia grade by ASA		
I	9	11.2
II	23	28.8
III	30	37.5
IV	18	22.5
V	0	0
Volume of perioperative blood transfusion (*n* = 24), mL	300 (100–1000)	
Duration of operation, min.	150 (40–340)	
Duration of ICU in-hospital stay (*n* = 15), d.	3 (1–9)	
Duration of postoperative in-hospital stay, d.	6 (3–15)	
Duration of total in-hospital stay, d.	9 (6–20)	
Postoperative complications (*n* = 21)		
Pancreatic fistula	10	12.5
Intra-abdominal infection	7	8.8
Delayed gastric emptying	4	5.0
Intra-abdominal hemorrhage	2	2.5
Reoperation	1	1.3
In-hospital death	0	0
Postoperative medical therapy (*n* = 34)		
Novel molecular targeting treatment	14	17.5
Traditional chemotherapy	20	25
Patient prognosis		
Follow-up time, mon	58.3 (9.7–182.6)	
Out of contact	10	12.5
Dead at follow-up (*n* = 70)	39	55.7
Accumulative 5-year OS	29.7%	
MST, months	49.2 (95% CIs: 41.8–56.5)	

Abbreviations: G: grading; p-NETs: pancreatic neuroendocrine tumors; ASA: American Society of Anesthesiologists; ICU: intensive care unit; OS: overall survival; MST: median survival time. [A]: Referring to resections with negative surgical margins, both grossly and microscopically. [B]: Referring to cholangiojejunostomy, gastrojejunostomy, etc. with simultaneous biopsy when the local lesion was unresectable or distant metastasis was detected during the intraoperative exploration.

As Table 2 listed, the median follow-up time of our study was 58.3 months (ranging from 9.7 months to 182.6 months). When the follow-up ended, 10 patients were out of

contact (12.5%). There were 39 deaths related to the disease progression (55.7%). The accumulative 5-year OS of G3 p-NETs was 29.7% (Figure 1), with an MST of 49.2 months (95% CIs: 41.8 months–56.5 months). The accumulated 5-year OS from current AJCC Stage I to Stage IV was 100.0%, 31.3%, 17.1% and not applicable (NA), respectively (Figure 2). Patients classified using the current AJCC Stage I had better survival than those in Stage II ($p = 0.003$), Stage III ($p = 0.006$) and Stage IV ($p < 0.001$), as well as when comparing Stage II with Stage IV ($p < 0.001$) or comparing Stage III with Stage IV ($p < 0.001$). However, the survival comparison between the current AJCC Stage II and Stage III was not significant ($p = 0.172$). The 5-year OS for the proposed mTNM Stage I, Stage II, Stage III and Stage IV was respectively 100%, 39.1%, 15.6% and NA (Figure 3). Patients defined as mTNM Stage I had better survival than those at Stage III ($p = 0.005$) and Stage IV ($p < 0.001$), as well as those at Stage II compared with Stage III ($p = 0.016$) and Stage IV ($p < 0.001$). Meanwhile, the comparisons of OS between Stage I and Stage II or between Stage III and Stage IV were both statistically significant ($p = 0.004$ and $p < 0.001$, respectively).
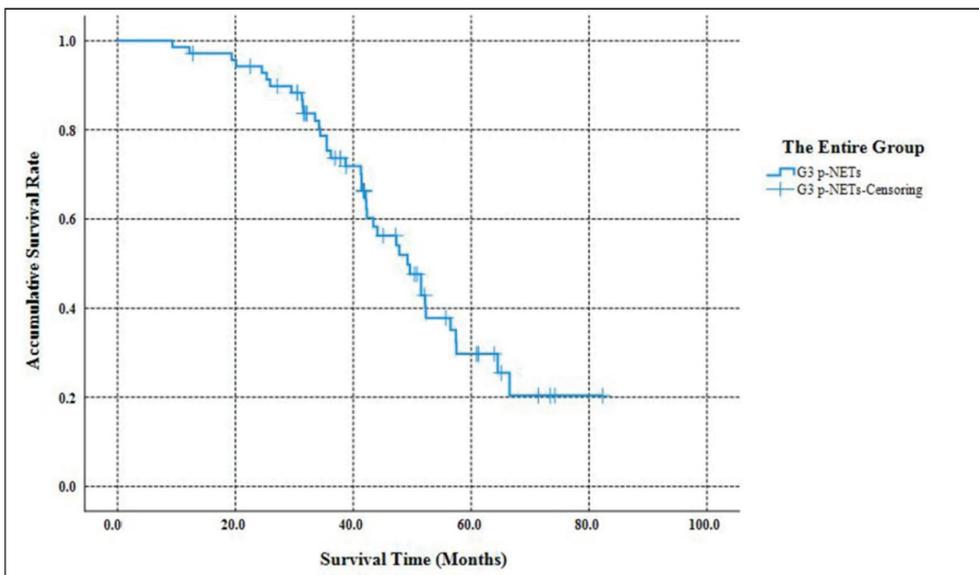
Figure 1. Kaplan–Meier estimates for the OS of the entire group of G3 p-NETs defined by the WHO 2017 grading classification.

As listed in Table 3, patients' gender ($p = 0.236$) and age ($p = 0.121$), tumor location ($p = 0.415$), incidental diagnosis ($p = 0.478$), mitotic rate ($p = 0.125$), harvested lymph nodes ($p = 0.512$), postoperative complications ($p = 0.517$), duration of operation ($p = 0.343$) and postoperative in-hospital stay ($p = 0.952$) were demonstrated to have no notable impacts on the OS of G3 p-NETs, while the survival analyses referring to tumor type ($p = 0.012$), tumor diameter ($p = 0.016$), Ki-67 index ($p = 0.035$), necrosis ($p = 0.027$), operation classification ($p < 0.001$), postoperative medical therapy ($p = 0.042$), current AJCC 8th staging system ($p < 0.001$) and proposed mTNM staging system ($p < 0.001$) were statistically significant in univariate analyses. Using multivariate analyses in different Cox regression models, we concluded that only operation classification ($p = 0.031$ and $p = 0.027$, respectively), current AJCC 8th staging system ($p = 0.017$) and proposed mTNM staging system ($p = 0.032$) were independent predictors for the OS of G3 p-NETs. Meanwhile, the 95% CIs of the proposed mTNM staging system (0.626–8.217) were smaller than those of the current AJCC 8th staging system (0.329–10.013), indicating a relatively more accurate predictive ability.
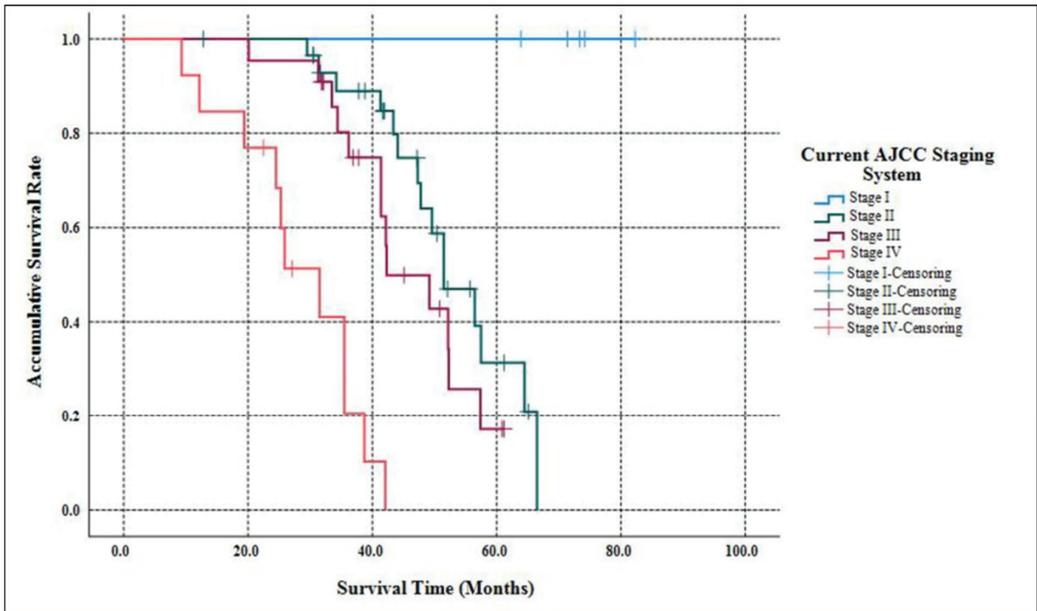
Figure 2. Kaplan–Meier estimates for the OS of G3 p-NETs according to the current AJCC 8th edition staging system originally proposed for G1/G2 p-NETs.
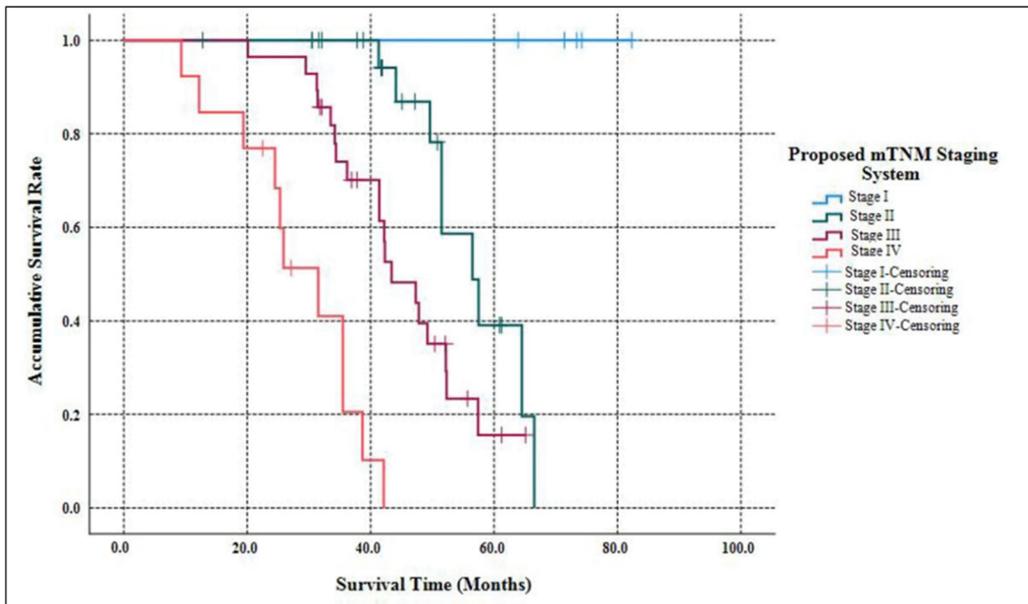
Figure 3. Kaplan–Meier estimates for the OS of G3 p-NETs, according to the newly proposed mTNM staging system primarily designed for G1/G2 p-NETs.

Table 3. Univariate and multivariate analyses of prognostic factors for predicting the OS of G3 p-NETs in our study.

Factor	Univariate Analysis		Multivariate Analysis	
	HR (95% CIs)	*p*	HR (95% CIs)	*p*
Patients' gender				
Male [A]				
Female	1.244 (0.864–1.653)	0.236		
Patients' age				
<50 [B]				
≥50	0.931 (0.512–1.349)	0.121		
Tumor location				
Head/uncinate				
Body/tail	1.012 (0.626–1.431)	0.415		
Tumor type				
Functional				
Non-functional1	1.425 (0.712–2.324)	**0.012**	1.034 (0.523–1.517)	0.512 [C]
			1.213 (0.671–1.642)	0.214 [D]
Incidental diagnosis				
No				
Yes	0.973 (0.5157–1.436)	0.478		
Tumor diameter				
<4.5				
≥4.5	1.479 (0.762–2.962)	**0.016**	0.783 (0.361–1.452)	0.257
			0.981 (0.382–1.901)	0.538
Ki-67 index				
<28				
≥28	2.069 (0.982–4.123)	**0.035**	1.253 (0.564–2.122)	0.873
			0.902 (0.468–2.093)	0.435
Mitotic rate				
<22				
≥22	1.214 (0.614–1.892)	0.125		
Necrosis				
Absent				
Present	3.024 (1.243–7.146)	**0.027**	1.441 (0.684–2.679)	0.137
			0.993 (0.414–1.983)	0.561
Harvested lymph nodes				
<8				
≥8	1.001 (0.425–1.458)	0.512		
Operation classification				
Resection				
Palliative	2.221 (1.329–4.186)	**<0.001**	1.523 (0.723–3.215)	**0.031**
			1.734 (0.757–3.953)	**0.027**
Duration of operation				
<150				
≥150	1.275 (0.546–2.325)	0.343		
Duration of postoperative in-hospital stay				
<6				
≥6	1.241 (0.547–1.874)	0.952		
Postoperative complications				
No				
Yes	0.893 (0.434–2.082)	0.517		
Postoperative medical therapy				
No				
Yes	2.145 (0.783–3855)	**0.042**	1.314 (0.424–2.325)	0.518
			1.211 (0.384–1.924)	0.892
Current AJCC 8th staging system [E]				
Stage I/II				
Stage III/IV	3.124 (1.332–5.478)	**<0.001**	5.363 (0.329–10.013)	**0.017**
			NA	
Proposed mTNM staging system [E]				
Stage I/II				
Stage III/IV	3.954 (0.996–8.326)	**<0.001**	NA	
			3.213 (0.626–8.217)	**0.032**

[A]: This related factor was regarded as a reference in the Cox analysis. [B]: The value of "median" for quantitative variables was regarded as the cut-off in the Cox analysis. [C]: The upper results of the multivariate analysis for each factor were demonstrated in Cox hazard models with the current AJCC 8th staging system. [D]: The bellow results of the multivariate analysis for each factor were demonstrated in Cox hazard models with the proposed mTNM staging system. [E]: The potential prognostic value of two different systems was demonstrated in separate Cox hazard models. Abbreviations: OS: overall survival; G: grading; p-NETs: pancreatic neuroendocrine tumors; AJCC: American Joint Committee on Cancer; mTNM: modified tumor–node–metastasis; HR: hazard ratio; CIs: confidence interval; NA: not applicable.

4. Discussion

As we knew, G1/G2 p-NETs were regarded as well-differentiated, while G3 p-NECs were poorly-differentiated according to the grading classification by ENETS and the WHO [5,6]. However, subsequent studies revealed that, although all poorly-differentiated

neuroendocrine carcinomas had a high proliferation rate, not all p-NENs with a proliferation rate above 20% were poorly-differentiated, indicating the heterogeneity of G3 p-NECs [8–12]. Referring to both the tumor morphology and Ki-67 index, the WHO incorporated a new subcategory of "well-differentiated high-grade tumors (i.e., G3 p-NETs)" into the well-differentiated p-NETs category in its 2017 *Classification of the Tumors of Endocrine Organs* [14], which was proven to be superior to the WHO 2010 criteria [20]. Nevertheless, the clinical features of G3 p-NETs have not yet been well documented.

In the present research, we made an in-depth analysis with regard to the surgical outcomes, prognostic factors and staging systems of G3 p-NETs. We revealed that the baseline demographics and tumor characteristics of G3 p-NETs, such as patients' gender and age, tumor location and type, were in agreement with our previous results [16,20]. As we demonstrated in Table 3, patients with non-functional G3 p-NETs showed significantly worse survivals than those with functional tumors ($p = 0.012$), while the other factors had no obvious influence on the OS of G3 p-NETs. However, tumor type could not be a significant prognostic factor for the OS of G3 p-NETs ($p = 0.512$ and $p = 0.214$, respectively), as we previously demonstrated [16]. Moreover, while the CgA was expressed in all G3 p-NETs in the immunohistochemical examinations, we failed to test the plasma CgA values in the present study due to our limited technologies. Massironi et al., reported that plasma CgA had a significant prognostic relevance for patients with gastroenteropancreatic neuroendocrine neoplasms [26], while the prognostic value of plasma CgA for patients with G3 p-NETs still needed to be validated in future studies.

Accumulated studies reported that p-NENs at the lower end of the G3 range might, in fact, be well-differentiated with elevated Ki-67 proliferative rates and better survivals [8,9], which intrinsically prompted the formation of the WHO's 2017 grading classification [14]. However, the role of the Ki-67 index for the new group of G3 p-NETs remains unknown due to the currently limited data in the literature. In 2018, Mizuno et al. [27] identified 10 patients with G3 p-NETs, with a median Ki-67 index of 35% (ranging from 20% to 90%), although the impact of Ki-67 on the survival of G3 p-NETs was not evaluated. Recently, de Mestier et al. [28] reported 74 patients with digestive well-differentiated G3 neuroendocrine tumors (including 53 cases located in pancreas/duodenum), with a median Ki-67 index of 30% (ranging from 21% to 80%). Meanwhile, de Mestier et al. [28] demonstrated that the Ki-67 index was not a significant predictor for the progression-free survival of these patients. In our study, the median Ki-67 index of this cohort was 28%, which was very close to the above reported data [27,28], as well as our previous results [16]. According to our validation, the Ki-67 index did indeed influence the prognosis of G3 p-NETs ($p = 0.035$), but failed to be a significant prognostic factor for the patients' OS estimate ($p = 0.873$ and $p = 0.435$, respectively).

As reported [29,30], the molecular features and prognosis of G3 p-NETs largely differ from those of G3 p-NECs and are much closer to those of G2 p-NETs, while the most appropriate management for G3 p-NETs is currently undefined. Several studies suggested that G3 p-NETs should be treated as G2 p-NETs with respect to both surgical programs and systemic therapies [31–33]. Feng et al. [32] reported that the median survival was higher in patients undergoing surgery, while non-surgical management was a poor prognostic factor associated with reduced disease-specific survival in patients with G3 p-NETs. Yoshida et al. [33] revealed that surgical procedures for G3 p-NETs and G3 p-NECs should be considered separately, and that patients with G3 p-NETs could significantly benefit from surgical resection for both primary pancreatic tumors and selected metastatic disease. Meanwhile, the MST in Yoshida et al.'s research was lower than that in our report (33 months and 49.2 months, respectively), which could be explained by differences in the inclusive criteria in each cohort. What is more, we demonstrated that surgical resection was an independent and favorable predictor for the survival of G3 p-NETs ($p = 0.031$ and $p = 0.027$, respectively), which was consistent with the reports by Yoshida et al. [33]. Unfortunately, only six selected patients with metastatic disease in our study received radical resection accompanied by pancreatic surgery, making it difficult to evaluate its impact on patients' survival.

Surgery is the optimal and curative treatment for p-NENs, while drug therapy is also very important and effective in terms of systemic treatment [1,4]. Studies have proposed that molecular targeted drugs such as sunitinib and everolimus be recommended for patients with G1/G2 p-NETs, while platinum-based chemotherapies are the first-line drugs for all p-NENs except G1/G2 p-NETs [34]. However, there have been no standardized and well-recognized medical therapeutic schedules for G3 p-NETs. Mizuno et al. [27] reported that sunitinib was as effective for G3 p-NETs as for G1/G2 p-NETs, which could significantly improve both progression-free survival and OS by reducing the tumors' volume. Moreover, de Mestier et al. [28] revealed that adenocarcinoma-like and alkylating-based chemotherapies were the most effective treatments for advanced G3 neuroendocrine tumors regarding objective response and progression-free survival, while etoposide–platinum chemotherapy had poor efficacy in that setting. Our study enrolled 20 patients with postoperatively traditional chemotherapies and 14 patients with novel molecular targeting treatments. The changes of drug therapy for G3 p-NETs might be the result of the varied recognitions for this new subcategory of p-NENs. We demonstrated that postoperative medical therapy had notable impacts on the OS of G3 p-NETs ($p = 0.042$), although it could not be an independent predictor ($p = 0.518$ and $p = 0.892$, respectively). However, we failed to compare the impacts of traditional chemotherapies and molecular targeting treatments on the OS of G3 p-NETs due to their different drug schemes in the limited cases of this study.

The current AJCC 8th staging manual for p-NENs elucidates stratifying G1/G2 p-NETs and G3 p-NECs into different stages separately, while the most practical and appropriate staging system for G3 p-NETs remains unclear [15]. Although we previously demonstrated that G3 p-NETs might also be staged using the same AJCC system as the current one for G1/G2 p-NETs [16], this system has so far failed to distinguish prognosis among patients with Stage I vs. Stage II disease or Stage II vs. Stage III disease [17–20]. Recently, a new mTNM staging system on the basis of the current AJCC system was proposed and assessed for G1/G2 p-NETs [21], which was highly appraised [23,24], but not yet validated for G3 p-NETs. We hereby succeeded in defining G3 p-NETs into four stages using both the current AJCC staging system and the proposed mTNM approach. Furthermore, the current AJCC system failed to discriminate the survival difference between Stage II and Stage III ($p = 0.172$; Figure 2), as You et al., demonstrated [18], while notable differences with regard to the OS of G3 p-NETs were statistically offered between each mTNM stage (all $p < 0.05$; Figure 3). Meanwhile, although both systems were prognostic for predicting the OS of G3 p-NETs ($p = 0.032$ and $p = 0.017$, respectively), the 95% CIs of the mTNM staging system were smaller than that of the current AJCC system (0.626–8.217 and 0.329–10.013, respectively), indicating a potentially more accurate predictive ability. Our results of the comparisons between the applications of the mTNM system and the current AJCC approach to G3 p-NETs were similar to the validations of Zhang's study for G1/G2 p-NETs [21], suggesting that the newly proposed mTNM staging system was more suitable and practical for G3 p-NETs.

Our study had several limitations. First of all, it was a retrospective study from a single medical institution, leading to a small number of enrolled patients with a long follow-up time. Secondly, our study excluded those patients without surgery, which meant that comparisons could not be made between the clinical features and survival differences of patients with surgical treatments and non-surgical therapies. In addition, as we mentioned above, our study failed to compare the prognosis between the resection of primary tumors and metastatic diseases, as well as between traditional chemotherapies and molecular targeting treatments, due to our limited cases. Finally, the mTNM staging system for G1/G2 p-NETs was originally designed by Zhang et al. [21], while our study for G3 p-NETs was supplementary research for the indications of this new proposed system. Therefore, a multi-center, large-volume and prospective study is still needed to confirm our results.

5. Conclusions

According to our in-depth analyses, tumor type, Ki-67 index, necrosis and postoperative medical therapy had certain impacts on the survival of patients with G3 p-NETs, while surgical resection was an independent and favorable predictor for patients' OS estimate. Meanwhile, the newly proposed mTNM staging system was superior to the current AJCC system due to its better prognostic stratification and more accurate predicting ability for the OS of patients with G3 p-NETs, supporting its wider clinical use.

References

1. Ehehalt, F.; Saeger, H.D.; Schmidt, C.M.; Grutzmann, R. Neuroendocrine tumors of the pancreas. *Oncologist* **2009**, *14*, 456–467. [CrossRef]
2. Kloppel, G. Classification and pathology of gastroenteropancreatic neuroendocrine neoplasms. *Endocr. Relat. Cancer* **2011**, *18* (Suppl. S1), S1–S16. [CrossRef] [PubMed]
3. Nichholls, A.G. Simple adenoma of the pancreas arising from an island of Langerhans. *J. Med. Res.* **1902**, *8*, 385–395.
4. Ito, T.; Igarashi, H.; Nakamura, K.; Sasano, H.; Okusaka, T.; Takano, K.; Komoto, I.; Tanaka, M.; Imamura, M.; Jensen, R.T.; et al. Epidemiological trends of pancreatic and gastrointestinal neuroendocrine tumors in Japan: A nationwide survey analysis. *J. Gastroenterol.* **2015**, *50*, 58–64. [CrossRef] [PubMed]
5. Williams, E.D.; Siebenmann, R.E.; Sobin, L.H.; World Health Organization. Histological Typing of Endocrine Tumours. In *World Health Organization International Histological Classification of Tumours*, 2nd ed.; Springer: Berlin, Germany, 2000.
6. Rindi, G.; Klöppel, G.; Alhman, H.; Caplin, M.; Couvelard, A.; de Herder, W.W.; Eriksson, B.; Falchetti, A.; Falconi, M.; Komminoth, P.; et al. TNM staging of foregut (neuro) endocrine tumors: A consensus proposal including a grading system. *Virchows Arch.* **2006**, *449*, 395–401. [CrossRef] [PubMed]
7. Rindi, G.; Arnold, R.; Bosman, F.T.; Capella, C. Nomenclature and classification of neuroendocrine neoplasms of the digestive system. In *WHO Classification of Tumours of the Digestive System*, 4th ed.; Bosman, F.T., Carneiro, F., Hruban, R.H., Theise, N.D., Eds.; International Agency for Research on Cancer: Lyon, France, 2010; pp. 13–40.
8. Basturk, O.; Yang, Z.; Tang, L.H.; Hruban, R.H.; Adsay, V.; McCall, C.; Krasinskas, A.M.; Jang, K.-T.; Frankel, W.L.; Balci, S.; et al. The high-grade (WHO G3) pancreatic neuroendocrine tumor category is morphologically and biologically heterogeneous and includes both well-differentiated and poorly differentiated neoplasms. *Am. J. Surg. Pathol.* **2015**, *39*, 683–690. [CrossRef]
9. Sorbye, H.; Welin, S.; Langer, S.W.; Vestermark, L.W.; Holt, N.; Osterlund, P.; Dueland, S.; Hofsli, E.; Guren, M.G.; Ohrling, K.; et al. Predictive and prognostic factors for treatment and survival in 305 patients with advanced gastrointestinal neuroendocrine carcinoma (WHO G3): The NORDIC NEC study. *Ann. Oncol.* **2013**, *24*, 152–160. [CrossRef]

10. Tang, L.H.; Basturk, O.; Sue, J.J.; Klimstra, D.S. A practical approach to the classification of WHO grade 3 (G3) well differentiated neuroendocrine tumor (WD-NET) and poorly differentiated neuroendocrine carcinoma (PDNEC) of the pancreas. *Am. J. Surg. Pathol.* **2016**, *40*, 1192–1202. [CrossRef]

11. Chen, Y.; Yang, M.; Wang, W.G.; Huang, W.; Wang, L.; Tian, B. Long-term outcomes of World Health Organization G3 pancreatic neuroendocrine neoplasms: A retrospective study in combination of morphological and proliferative analysis. *Int. J. Clin. Exp. Pathol.* **2017**, *10*, 6385–6394.

12. Milione, M.; Maisonneuve, P.; Spada, F.; Pellegrinelli, A.; Spaggiari, P.; Albarello, L.; Pisa, E.; Barberis, M.; Vanoli, A.; Buzzoni, R.; et al. The clinicopathologic heterogeneity of grade 3 gastroenteropancreatic neuroendocrine neoplasms: Morphological differentiation and proliferation identify different prognostic categories. *Neuroendocrinology* **2017**, *104*, 85–93. [CrossRef]

13. Yang, M.; Zhang, Y.; Zeng, L.; Ke, N.-W.; Tan, C.-L.; Tian, B.-L.; Xiang, B.; Liu, X.-B. Survivals of patients with surgically treated and High-grade pancreatic neuroendocrine carcinomas: A comparative study between two American Joint Committee on Cancer 8th tumor-node-metastasis staging systems. *Eur. J. Surg. Oncol.* **2019**, *45*, 1054–1060. [CrossRef] [PubMed]

14. Klöppel, G.; Couvelard, A.; Hruban, R.H.; Komminoth, P.; Osamura, R.Y.; Perren, A.; Rindi, G. Tumours of the endocrine pancreas. In *WHO Classification of the Tumours of Endocrine Organs*, 4th ed.; Lloyd, R.V., Osamura, R.Y., Klöppel, G., Rosai, J., Eds.; International Agency for Research on Cancer: Lyon, France, 2017; pp. 175–207.

15. Brierley, J.D.; Gospodarowicz, K.; Wittekind, C. (Eds.) *TNM Classification of Malignant Tumours*, 8th ed.; Wiley Blackwell: Bognor Regis, UK, 2017.

16. Deng, B.Y.; Yang, M.; Wen, J.Y.; Hou, S.-Z.; Chen, Y.; Tian, B.-L.; Liu, X.-B.; Zhang, Y. Survivals of patients with pancreatic neuroendocrine carcinomas: An in-depth analysis by the American Joint Committee on Cancer 8th tumor-node-metastasis staging manual. *Medicine* **2020**, *99*, e18736. [CrossRef] [PubMed]

17. Li, X.; Gou, S.; Liu, Z.; Ye, Z.; Wang, C. Assessment of the American Joint Commission on cancer 8th edition staging system for patients with pancreatic neuroendocrine tumors: A surveillance, epidemiology, and end results analysis. *Cancer Med.* **2018**, *7*, 626–634. [CrossRef] [PubMed]

18. You, Y.; Jang, J.-Y.; Kim, S.C.; Yoon, Y.-S.; Park, J.S.; Cho, C.K.; Park, S.-J.; Yang, J.D.; Lee, W.J.; Hong, T.H.; et al. Validation of the 8th AJCC cancer staging system for pancreas neuroendocrine tumors using Korean nationwide surgery database. *Cancer Res. Treat.* **2019**, *51*, 1639–1652. [CrossRef]

19. Yang, M.; Zhang, Y.; Zeng, L.; Ke, N.-W.; Tan, C.-L.; Tian, B.L.; Xiang, B.; Liu, X.-B. Prognostic validity of the American Joint Committee on Cancer Eighth Edition TNM staging system for surgically treated and well-differentiated pancreatic neuroendocrine tumors: A comprehensive analysis of 254 consecutive patients from a large Chinese institution. *Pancreas* **2019**, *48*, 613–621.

20. Yang, M.; Zeng, L.; Ke, N.-W.; Tan, C.-L.; Tian, B.-L.; Liu, X.-B.; Xiang, B.; Zhang, Y. World Health Organization grading classification for pancreatic neuroendocrine neoplasms: A comprehensive analysis from a large Chinese institution. *BMC Cancer* **2020**, *20*, 906. [CrossRef]

21. Zhang, X.-F.; Xue, F.; Wu, Z.; Lopez-Aguiar, A.G.; Poultsides, G.; Makris, E.; Rocha, F.; Kanji, Z.; Weber, S.; Fisher, A.; et al. Development and Validation of a Modified Eighth AJCC Staging System for Primary Pancreatic Neuroendocrine Tumors. *Ann. Surg.* **2022**, *275*, e773–e780. [CrossRef]

22. Zhang, X.F.; Xue, F.; Dong, D.H.; Lopez-Aguiar, A.G.; Poultsides, G.; Makris, E.; Rocha, F.; Kanji, Z.; Weber, S.; Fisher, A.; et al. New nodal staging for primary pancreatic neuroendocrine tumors: A multi-institutional and national data analysis. *Ann. Surg.* **2021**, *274*, e28–e35. [CrossRef]

23. Al-Toubah, T.; Strosberg, J. Progress in TNM staging of pancreatic neuroendocrine tumors. *Hepatobiliary Surg. Nutr.* **2021**, *10*, 238–240. [CrossRef]

24. Nicola, F.; Francesca, S.; Emilio, B. Looking for the right TNM staging system for pancreatic neuroendocrine tumors. *Hepatobiliary Surg. Nutr.* **2021**, *10*, 382–384.

25. Crawley, F.P. The Limits of the Declaration of Helsinki. In *Address to Scientific Session*; World Medical Association General Assembly: Helsinki, Finland, 2012.

26. Mizuno, Y.; Kudo, A.; Akashi, T.; Akahoshi, K.; Ogura, T.; Ogawa, K.; Ono, H.; Mitsunori, Y.; Ban, D.; Tanaka, S.; et al. Sunitinib shrinks NET-G3 pancreatic neuroendocrine neoplasms. *J. Cancer Res. Clin.* **2018**, *144*, 1155–1163. [CrossRef]

27. de Mestier, L.; Lamarca, A.; Hernando, J.; Zandee, W.; Alonso-Gordoa, T.; Perrier, M.; Walenkamp, A.M.; Chakrabarty, B.; Landolfi, S.; Van Velthuysen, M.F.; et al. Treatment outcomes of advanced digestive well-differentiated grade 3 NETs. *Endocr. Relat. Cancer* **2021**, *28*, 549–561. [CrossRef]

28. Konukiewitz, B.; Schlitter, A.M.; Jesinghaus, M.; Pfister, D.; Steiger, K.; Segler, A.; Agaimy, A.; Sipos, B.; Zamboni, G.; Weichert, W.; et al. Somatostatin receptor expression related to TP53 and RB1 alterations in pancreatic and extrapancreatic neuroendocrine neoplasms with a Ki67-index above 20%. *Mod. Pathol.* **2017**, *30*, 587–598. [CrossRef]

29. Sorbye, H.; Baudin, E.; Borbath, I.; Pfister, D.; Steiger, K.; Segler, A.; Agaimy, A.; Sipos, B.; Zamboni, G.; Weichert, W.; et al. Unmet needs in high-grade gastroenteropancreatic neuroendocrine neoplasms (WHO G3). *Neuroendocrinology* **2019**, *108*, 54–62. [CrossRef] [PubMed]

30. Holmager, P.; Langer, S.W.; Kjaer, A.; Ringholm, L.; Garbyal, R.S.; Pommergaard, H.-C.; Hansen, C.P.; Federspiel, B.; Andreassen, M.; Knigge, U. Surgery in Patients with Gastro-Entero-Pancreatic Neuroendocrine Carcinomas, Neuroendocrine Tumors G3 and High Grade Mixed Neuroendocrine-Non-Neuroendocrine Neoplasms. *Curr. Treat. Options Oncol.* **2022**, *23*, 806–817. [CrossRef]

31. Feng, T.; Lv, W.; Yuan, M.; Shi, Z.; Zhong, H.; Ling, S. Surgical resection of the primary tumor leads to prolonged survival in metastatic pancreatic neuroendocrine carcinoma. *World J. Surg. Oncol.* **2019**, *17*, 54. [CrossRef]
32. Yoshida, T.; Hijioka, S.; Hosoda, W.; Ueno, M.; Furukawa, M.; Kobayashi, N.; Ikeda, M.; Ito, T.; Kodama, Y.; Morizane, C.; et al. Surgery for pancreatic neuroendocrine tumor G3 and carcinoma G3 should be considered separately. *Ann. Surg. Oncol.* **2019**, *26*, 1385–1393. [CrossRef]
33. Pavel, M.; O'Toole, D.; Costa, F.; Capdevila, J.; Gross, D.; Kianmanesh, R.; Krenning, E.; Knigge, U.; Salazar, R.; Pape, U.-F.; et al. ENETS consensus guidelines update for the management of distant metastatic disease of intestinal, pancreatic, bronchial neuroendocrine neoplasms (NEN) and NEN of unknown primary site. *Neuroendocrinology* **2016**, *103*, 172–185. [CrossRef]
34. Stelwagen, J.; de Vries, E.G.E.; Walenkamp, A.M.E. Current treatment strategies and future directions for extrapulmonary neuroendocrine carcinomas: A review. *JAMA Oncol.* **2021**, *7*, 759–770. [CrossRef]

Review

Circulating Neuroendocrine Tumor Biomarkers: Past, Present and Future

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Abstract: Neuroendocrine tumors are a heterogeneous group of neoplasms originating from the diffuse endocrine system. Depending on primary location and hormonal status, they range in terms of clinical presentation, prognosis and treatment. Functional tumors often develop symptoms indicating an excess of hormones produced by the neoplasm (exempli gratia insulinoma, glucagonoma and VIPoma) and can be diagnosed using monoanalytes. For non-functional tumors (inactive or producing insignificant amounts of hormones), universal biomarkers have not been established. The matter remains an important unmet need in the field of neuroendocrine tumors. Substances researched over the years, such as chromogranin A and neuron-specific enolase, lack the desired sensitivity and specificity. In recent years, the potential use of Circulating Tumor Cells or multianalytes such as a circulating microRNA and NETest have been widely discussed. They offer superior diagnostic parameters in comparison to traditional biomarkers and depict disease status in a more comprehensive way. Despite a lot of promise, no international standards have yet been developed regarding their routine use and clinical application. In this literature review, we describe the analytes used over the years and cover novel biomarkers that could find a use in the future. We discuss their pros and cons while showcasing recent advances in the field of neuroendocrine tumor biomarkers.

Keywords: neuroendocrine tumors; biomarkers; chromogranin A; neuroendocrinology; neuroendocrine neoplasms; microRNA; NETest; 5-HIAA

1. Introduction

Neuroendocrine tumors (NETs) are a diverse group of neoplasms. They are made from diffuse neuroendocrine system cells, which are present throughout the human body. The prevalence of neuroendocrine tumors ranges between 2.5 and 8.35 cases per 10,000, with incidence rates rising in recent years [1–3]. NETs fulfill the rare disease criteria according to the Orphan Drug Act (a condition affecting less than 200,000 people in the United States). Neuroendocrine Tumors, along with a second subunit, Neuroendocrine Carcinomas (NECs), are a part of a group named Neuroendocrine Neoplasms (NENs), as per WHO nomenclature [4]. Despite a similar origin from neuroendocrine tissue, both of them have their own distinct morphological features and genomic signatures. NETs can be both low- and high-grade, whereas NEC are high-grade by definition. In order to distinguish NETs from NECs, pathologists utilize tissue biomarkers of neuroendocrine lineage such as synaptophysin, chromogranin A and somatostatin receptors, some of which can also be used as circulating biomarkers [5]. Due to significant differences between both groups in terms of clinical presentation, applicable biomarkers and the natural course of the disease, this review focuses mainly on NETs. Depending on their embryonic origin (from which part of the primary gut tube the tumor originates from), NETs can be divided into three groups: foregut, midgut and hindgut, each with their own distinct characteristics [6].

The primary location of the tumor, and which part of the primary gut tube the neoplasm stems from, affects the application and clinical utility of different biomarkers. For instance, 5-hydroxyindoleacetic acid (5-HIAA) shows a higher sensitivity in midgut NENs than in pancreatic NENs, whereas the expression of chromogranin A (CgA) is lower in hindgut tumors, compared to midgut and foregut [7]. Biomarkers aside, embryonic origin directly affects the diagnostic and treatment procedures, as patients diagnosed with foregut NENs should undergo screening for MEN-1 syndrome [8]. Table 1 presents examples of primary tumor locations falling into each category.

Table 1. Examples of neuroendocrine tumors' primary locations of different embryonic origin.

Foregut	Midgut	Hindgut
Thymus	Jejunum	Distal 1/3 of transverse colon
Esophagus	Appendix	Descending colon
Bronchus	Ileum	Sigmoid colon
Lung	Ascending colon	Rectum
Stomach	Proximal 2/3 of transverse colon	
Pancreas		
Duodenum		

NETs can be divided based on their ability to release hormones (functional tumors) or not (non-functional tumors). Neoplasms producing clinically insignificant amounts of hormones also fall into the latter bracket. In the case of functional tumors, hormones released into the circulation allow for utilizing them as biomarkers, as shown on Table 2.

Table 2. Functional pancreatic NET and corresponding specific biomarkers.

Type of Tumor	Secreted Hormone	Incidence (New/100,000/Year) [9]
Insulinoma	Insulin	1–32
Gastrinoma	Gastrin	0.5–21.5
VIPoma	Vasoactive Intestinal Peptide	0.05–0.2
Glucagonoma	Glucagon	0.01–0.1
Somatostatinoma	Somatostatin	Rare < 0.1
GRHoma	GH-releasing hormone	Rare
Ghrelinoma	Ghrelin	Unknown (>100 cases described)
ACTHoma	ACTH	Rare
Pancreatic NET causing Carcinoid Syndrome	Serotonin	Rare (<100 cases)
Pancreatic NET causing hypercalcemia	PTHrP (Parathyroid Hormone-related Peptide)	Rare

Additionally, excess concentration of a given hormone is linked with symptoms specific to the disease. For example, insulinoma, an insulin-producing tumor most commonly found in the pancreas, typically presents with hypoglycemic episodes [10]. These characteristics allow for a relatively quick and accurate diagnosis, however, there are certain limitations. Functional tumors constitute a minority of all NENs (10–40%), with some of them being extremely rare (<100 cases described worldwide) [8]. Clinical manifestations may change over the course of the disease and there are a number of factors that cause similar symptoms or test results (for instance, exogenous insulin intake or Hirata's disease mimic insulinoma). Hormonal testing should be guided by the presence of symptoms in an individual; screening for the disease in patients with asymptomatic disease isn't required [11]. On the contrary, non-functional tumors lack a specific biomarker or the spectrum of symptoms that would allow for a quick diagnosis. The patient might not spot any manifestations of the disease until the lesion starts infiltrating nearby tissue or the

metastases start impairing the function of distant organs. In fact, 12–22% of patients present at the metastatic stage, despite the slow growth of neuroendocrine tumors [3].

Over the years, researchers and physicians tried to find molecules that could help diagnose neuroendocrine tumors early, improving patient prognosis. Due to the heterogeneous nature of neuroendocrine tumors, the search for a one-for-all analyte has so far been unsuccessful. This article aims to review circulating biomarkers used in daily clinical practice over the years, as well as to discuss the latest findings regarding the potential future biomarkers.

2. Materials and Methods

Upon forming the topic of the review, a thorough literature search was conducted. Initially, the guidelines of selected endocrinological societies were analyzed (Polish Society of Endocrinology, Polish Network of Neuroendocrine Tumors, European Society for Medical Oncology, European Neuroendocrine Tumor Society, North American Neuroendocrine Tumor Society). Afterwards, the PubMed database was searched using general terms: "neuroendocrine tumors", "neuroendocrine neoplasms", "neuroendocrine tumor biomarkers", "neuroendocrine neoplasms biomarkers", "NET biomarkers", "NEN biomarkers" and "neuroendocrine biomarkers". A second detailed search was conducted after a review of the initial results, focusing on the substances that showed the most merit in the guidelines and analyzed papers. Terms screened for included: "chromogranin A", "chromogranin B", "granins", "5-hydroxyindoleacetic acid", "5-HIAA", "neuron-specific enolase", "NSE", "NETest", "microRNA", "Circulating tumor cells" and "CTC", as well as variations of the searches above combining the terms with the words "biomarkers", "NET" and "neuroendocrine". The alternate spelling of certain words was accounted for (tumor/tumour, neurospecific/neuro-specific/neuron-specific). Based on the results of the searches mentioned above, a manuscript was drafted. When citing original research, the number of patients involved and methodology was taken into account. In certain topics (namely CTC and miRNA), the number of published original papers remains low because of their novel status and recent discoveries, presenting a limitation of the review. After the verification of search results, titles and abstracts, a thorough analysis of 265 selected papers was conducted. The reference lists of selected papers were also analyzed and 25 additional relevant articles were found. In total, 163 papers were chosen for the review. Included in the total number were 6 additional articles suggested by the reviewers after the first round of peer-review and 7 abstracts from the 19th Annual ENETS conference.

3. Discussion

3.1. Granins

In 1967, Blaschko et al. described the soluble proteins found in bovine secretory granules, which they named chromogranins [12]. Some notable members of that group, discovered in later years, include chromogranins A (CgA) and B (CgB, also called secretogranin I), and secretogranins II (which used to be called chromogranin C), III and IV [13]. Since their discovery, numerous articles have been published describing their role in neuroendocrine secretion [14–17]. Elevated bodily fluid concentrations of different granins (most notably CgA) among patients with hormonally active neoplasms have been some of the most important observations established in that research and with far-reaching clinical implications. Subsequently, their role as a potential biomarker of hormonally active neoplasms (e.g., NETs, pheochromocytoma, medullary thyroid cancer and pituitary gland tumors) has been analyzed [18–20].

3.1.1. Chromogranin A

Ever since its discovery over 50 years ago, chromogranin A, a hydrophilic glycoprotein made up of 439 amino acids, remains the most widely used NET biomarker in clinical practice [21]. It is present in most neuroendocrine cells, as well as in neuroendocrine tumor cells, most notably midgut and pancreatic neoplasms [22]. It has been a staple in NET

diagnostics over the years, as noted by the guidelines from numerous scientific societies: European Neuroendocrine Tumor Society (ENETS) [23], North American Neuroendocrine Tumor Society (NANETS) [24], Polish Network of Neuroendocrine Tumours [8]. CgA concentration correlates with tumor burden; the highest values are observed in metastatic NETs [24]. Depending on the type of the tumor and location, sensitivity and specificity range between 68–81% and 56–100%, respectively [25–27]. Similarly to 5-HIAA, its sensitivity and specificity differs depending on the location; midgut tumors express CgA most often, foregut and hindgut less so [7]. Nobels et al. demonstrated that an elevated CgA is a valuable marker in patients with gastrinomas, pheochromocytomas, carcinoid tumors and non-functioning pancreatic NETs. Elevated CgA levels were found in 100%, 89%, 80% and 69%, respectively [28]. A high sensitivity of CgA in gastrinoma makes it useful for a post-treatment follow-up [29]. Additionally, CgA showcases a greater utility in monitoring the progression of the disease and treatment response than as a diagnostic biomarker, as revealed by a 2018 meta-analysis on the subject, and increased values of CgA can predate radiological progression or tumor recurrence [30–32]. Recent meta-analysis of bronchopulmonary Neuroendocrine Neoplasms (bpNEN) showed sensitivity of as little as 35%, with 94% specificity [33,34]. Moreover, CgA concentration correlates with tumor burden; the highest values are observed in metastatic NETs [24], in which the specificity of 100% and sensitivity between 78 and 80% have been reported [25]. The 2015 ENETS guidelines noted the lack of systematic empirical evidence for use of CgA in bpNEN [35]. In the wake of recent research, current guidelines state that treatment decisions should not be based solely on CgA results [11].

Despite relatively good sensitivity and specificity in certain tumors, CgA has some flaws. There are no standards available regarding testing and there are significant differences between the available assays (CgA can be measured in plasma and serum, using ELISA, IRMA and RIA methods). It is therefore recommended to use the same test (preferably in the same laboratory), when comparing results [8,36]. It is noteworthy that several factors might influence CgA concentration. Most common conditions include atrophic gastritis, Helicobacter pylori infection, kidney failure, liver cirrhosis, inflammatory bowel diseases, and other non-neuroendocrine neoplasms [37,38]. Additionally, certain medications may cause false-positive results by increasing gastrin secretion, namely proton pump inhibitors and H2-receptor antagonists [39–41]. In order to adequately evaluate CgA level, it is advised to withdraw potentially interfering medication at least 2 weeks before the testing [42,43].

3.1.2. Chromogranin B and Pancreastatin

Other granins such as CgB have also been researched as potential biomarkers, however their testing availability and, therefore, their clinical usefulness is limited [8]. Pancreastatin—a product of enzymatic cleavage of CgA—has shown to retain similar sensitivity and specificity to CgA, while being unaffected by PPI treatment [44,45]. Elevated concentrations of pancreastatin correlate with a shorter progression-free survival (PFS) and the overall survival (OS) of patients with pancreatic and small bowel NETs, which makes it a potential prognostic biomarker [46]. It seems to be especially useful in metastatic disease and recent data suggests that it compares better to CgA in detecting the progression of midgut NETs [47,48].

3.2. 5-Hydroxyindoleacetic Acid

5-hydroxyindoleacetic acid (5-HIAA), a serotonin metabolite, has been one of the longest used biomarkers in neuroendocrine tumors, since 1956 [49]. Serotonin is produced by enterochromaffin cells, most commonly located in the small intestine. It serves a purpose in regulating gastrointestinal tract motility [50]. Elevated levels of serotonin can be observed in neuroendocrine tumors, most commonly of midgut origin. Serotonin-secreting neuroendocrine tumors manifest as carcinoid syndrome. Originally, the term carcinoid was invented by Oberndorfer in 1907 and has been used to describe all NETs [51]. Currently

use of the term "carcinoid" is discouraged, due to the confusing terminology applied to it over the years. Serotonin is produced by 70% of all neuroendocrine tumors, with the percentage of serotonin positive gastric and pulmonary NETs reaching as low as 10–35%. Monitoring serotonin itself is challenging, due to fluctuations in its secretion over time as well as differences between individuals, therefore its metabolites, such as 5-HIAA, are preferred [52]. 5-HIAA can be measured both in serum and urine, although the latter is more broadly used. Urine samples need to be collected over a 24 h period, protected from light and added with an acidic compound to ensure stability [53]. The sensitivity of 5-HIAA in diagnosis and monitoring is quite low, around 35%, and strongly depends on serotonin secretion [54]. 5-HIAA urine concentration has shown a positive correlation with the severity of carcinoid syndrome [55]. Higher values are also observed in patients with metastatic midgut NETs, compared to non-metastatic patients, notably with liver metastases. Moreover, 5-HIAA could be a marker of a biochemical response to somatostatin analog treatment and may be useful in the early detection of recurrence post-surgery [21]. Despite a specificity of up to 100% in some trials [25], there are several factors limiting 5-HIAA use in daily clinical practice. Tryptophan-rich food, such as peanuts, bananas, chocolate, coffee and tea, as well as certain medication (e.g., diazepam and phenobarbital), might lead to false-positive results, therefore patients undergoing tests need to adhere to dietary restrictions [56]. In addition, 24 h urine collection is impractical, when compared to liquid biopsy due to a prolonged testing period and the requirement of additional equipment and preparation. The clinical usefulness of 5-HIAA is restricted to serotonin-producing tumors (i.e., manifesting as carcinoid syndrome), which applies to just a fraction of neoplasms.

3.3. Pancreatic Polypeptide, Neuropeptide Y and Peptide YY

Another circulating biomarker, described in literature as secreted by an NEN, is pancreatic polypeptide (PP). It belongs to the same group of peptides as Peptide YY (PYY) and neuropeptide Y (NPY). PP is a 36-amino-acid molecule involved in the regulation of the digestive tract function and food metabolism (i.e., increasing hepatic insulin sensitivity) [57]. Used on its own, PP has a low sensitivity of 41–63% for pNET and 18–53% for gastrointestinal NET [58]. Higher levels are associated with pancreatic tumors and metastatic disease. When used together with CgA, the test can detect NEN with a sensitivity of 84–96% [59]. Peptide YY is very similar to PP, with 18 of its 36 amino acids located in the same positions. PYY cells were found in gastrointestinal NEN tissue, most commonly in rectal NEN, where its presence has been associated with a worse prognosis [60,61]. The data on its use as a circulating biomarker are lacking. Another 36-amino-acid-long peptide is Neuropeptide Y. The elevated plasma levels of NPY have mostly been the focus of research in pheochromocytomas, neuroblastomas and gangliomas [62,63]. In one study by Allen et al., elevated levels of NPY were present in 6 out of 22 gastrointestinal NETs [64]. Whereas PP has some potential applications as a circulating NET biomarker, the utility of PYY and NPY is limited.

3.4. Neuron-Specific Enolase

In 1965, Moore and McGregor discovered a protein currently known as neuron-specific enolase (NSE) [65]. NSE is a glycolytic enzyme present in neurons and neuroendocrine cells in the central and peripheral nervous system. Elevated concentrations of NSE in body fluids can be found not only in septic shock and post-traumatic states, but also in conditions associated with cell proliferation, such as neoplasms [66,67]. The latter property was hoped to be useful in detecting NETs, however, research shows that NSE is elevated in just 19% of G1NET and 54% of G2NET cases. Therefore, it seems to be unreliable as a single diagnostic biomarker for well-differentiated tumors, however it can be of added value to CgA in G2NET cases [8,68]. NSE concentrations are significantly higher in NECs with a sensitivity of 63% in large cell neuroendocrine carcinoma (LCNEC) and 62% in small cell neuroendocrine carcinoma (SCNEC) [68]. Moreover, NSE may be useful as a predictor

of long-term survival in NEN cases and, thanks to its dependence on cell turnover, it is associated with malignant forms with a higher grading [69].

3.5. NETest

In 2007, a National Cancer Institute summit meeting on NETs was held. It was deemed that the currently available biomarkers have severe limitations and it is crucial to develop universal biomarkers for early diagnosis [70]. It has been widely discussed that molecular methods might describe an entity as dynamic and diverse as an NET in a much more adequate way than a single substance [71]. Therefore, in the last couple of years, researchers started moving towards complex multianalyte assays that utilize statistical algorithms.

An NETest is an example of one such method. It is based on evaluating a tumor's gene expression, i.e., its "biological signature". After performing a liquid biopsy and isolating the mRNA (messenger RNA), the cDNA (complementary DNA) is synthesized. Subsequently, PCR and gene analysis is performed, and the results are subjected to machine-learning algorithms. The resulting score is given on a scale from 0 to 100% (the normal score cut-off is 20%) [72].

The NETest has shown excellent diagnostic parameters in multiple trials, with both sensitivity and specificity exceeding 90% [73–76]. In the multicenter study published in 2021, Modlin et al. analyzed two cohorts of patients over 5 years. The first group focused on the NETest evaluation and was made up of 1684 NETs compared with 731 controls, whereas the second group was comparing an NETest with CgA and comprised 922 NETs versus 348 controls. In the described setting, the NETest identified 98% pheochromocytomas, 94% siNET, 91%panNET, 88%bpNET, 80% gastric NET and 79% NETs of the appendix. The NETest was more effective in diagnosing and monitoring NETs than CgA [77]. In a different trial, an NETest was able to detect progression 1 year before imaging methods [78]. Unlike CgA, factors such as PPI treatment and gastritis have no bearing on the results [79].

Overall, the NETest fits the criteria of an optimal biomarker thanks to its outstanding diagnostic properties, prognostic and predictive value that outperforms traditional analytes [80–83]. Among largely promising results, the NETest too has some potential downsides. Its cost-effectiveness is relatively unknown, and the question remains whether it can be widely introduced. On top of that, there are very few laboratories that are able to perform NETest analysis (i.e., Wren Laboratories in the USA and Sarah Cannon Molecular Diagnostics in Great Britain) [83].

3.6. microRNA

microRNAs (miRNA) are a group of small (22 nucleotides in length on average), non-coding RNA molecules that promote or suppress posttranscriptional gene expression [84]. Despite being discovered in 1993, their clinical applications only started gaining traction in the last few years [85]. miRNAs can be identified both in solid tissue as well as in body fluids (inter alia plasma, serum, saliva, CSF and urine). They can be secreted in autocrine, paracrine and endocrine ways (although the exact mechanisms are unknown) [86]. Such properties allow for an identification using a liquid biopsy and potentially making them useful as disease biomarkers [87]. Altered miRNA levels in body fluids are associated with numerous diseases (cardiovascular, gastrointestinal, renal, psychiatric, neoplasms etc.) [88–90]. In cancer, miRNAs can promote metastases, regulate angiogenesis and cell metabolism, as well as influence immune evasion and the response to certain treatment methods [87]. It is clear that miRNA dysregulation plays a crucial role in carcinogenesis and understanding the processes behind it might improve diagnosis and the treatment of oncological patients in the future [91].

miRNA have been extensively researched in most common neoplasms, e.g., ovarian cancer, lung cancer and colorectal cancer [92–94]. In comparison, little is known about circulating miRNA in NETs. The altered expression of over 100 miRNAs have been described in NETs [95]. So far, no universal target molecule for NETs has been identified, possibly

due to the diverse nature of neuroendocrine neoplasms and the fact that many miRNAs are tissue-specific [96]. Moreover, different molecules seem to be expressed in blood and tumor tissue, although some can be detected in both compartments [97]. Malczewska et al. summarized in their systematic review that in panNETs, miR-1290 is absent in tumor tissue, while miR-21 and MiR-133a seem to be present in both. In siNET, miR-7-5p, miR-31, miR-96, miR-133a, miR-182, miR-183, miR-196a and miR-215 can be traced in both blood and tumor tissue, while circulating miRNAs include additionally miR-21, miR-22, miR-150, miR-200a, miR-21, miR-133a and miR-144. Only miR-21 and miR-133a have been described as circulating miRNAs in both locations (the former also presents in lungs) [98].

Li et al. analyzed over 700 circulating miRNAs aiming to differentiate pancreatic cancer from NETs and benign pancreatic conditions. In that setting, the expression of miR-1290 was higher in the pancreatic cancer group vs. the NET group (81% sensitivity and 69% specificity), although no comparison has been made between NETs and other conditions. Several other miR showed statistically significant results (miR-628-3p, miR-550 and miR-1825), however, their diagnostic parameters were of lower value than miR-1290 [99]. Additionally, miR-375-3p distinguishes a low-grade lung NET from non-neuroendocrine lung tumors showing over 90% sensitivity and specificity [100]. miR-375 and miR-133a have been discussed as a biomarker of patient survival due to the down-regulation in tumor metastases of siNET, however, both as tumor tissue biomarkers) [101,102]. miR-375 seems to be particularly interesting, as it has been localized in enteroendocrine cells and has been described as an endocrine system modulator and marker of neuroendocrine differentiation [103,104]. miR-29b is a member of the miR-29 family, which has been researched as a biomarker for several cancers, including lung and ovary [105]. Özdirik et al. described a correlation between miR-29b and CgA levels, though no relation to OS has been shown [106]. Recently, the overexpression of 13 selected circulating miRNAs has been described in NENs and medullar, in comparison to healthy subjects. It was the first study in which a set of circulating miRNAs was identified that could represent a tumor signature for NEN diagnostics [107].

An expert consensus suggests that circulating miRNAs will be of use as a NET biomarker. However, as with most multianalytes, due to their complex nature, any potential tests will have to be based on mathematical algorithms in order to make them clinically viable [49]. A recent study by Nanayakkara et al. described a machine-learning algorithm utilizing a panel of 17 miRNAs that determines 15 NEN types with 98% accuracy. With further research, more refined algorithms will become available [96]. Another problem limiting potential clinical applications has been the unknown influence of treatment on miRNA expression. Somatostatin analogs change the patterns of circulating miRNA; the exact mechanisms of that process are poorly understood [108,109].

3.7. Circulating Tumor Cells

In neoplasms, as the tumor grows, certain cells split away from the lesion and enter circulation. These circulating tumor cells (CTC), if certain conditions are met, can settle down in a new location and form metastases [110]. The phenomenon has been described in the 19th century already by Thomas Ashworth, however, it took over 100 years until researchers began to understand the process behind it [111]. The first trials that focused on the isolation and identification of these cells were conducted in the late 20th century, and in 2004 CellSearch was approved by the United States Food and Drug Administration (FDA) as the first device for CTC analysis (at the time for use in breast cancer) [112]. Since then, multiple technologies were developed for detection in peripheral blood, utilizing CTC's distinct physical properties, immunoaffinity or direct analysis with fiber-optic arrays [113].

In NETs, CTC were detected for the first time in 2011. In a study published by Khan et al., 21% of panNETs and 43% of midgut NETs had detectable CTC. It is important to note however, that all subjects had metastatic disease at the time of the analysis [114]. In the 2013 follow-up study, 49% of patients in the group of 176 had at least one detectable CTC and the association between the presence of CTC and shorter PFS and OS has been

described [115]. Additionally, in 2016, research of 138 metastatic NET patients (primary sites included bronchopulmonary pancreas, midgut, hindgut and unknown primary location) was published. A low CTC count and CTC decrease post treatment had a favorable prognosis over a high CTC count, which correlated with a shorter OS [116]. Similar observations have been published in 2019 by Hsieh et al. in the study of Asian NET patients [117]. An effect of CTC presence on the effectiveness of somatostatin analog (SSA) treatment has been evaluated in the CALM-NET trial; patients with no detectable CTC might be more likely to respond positively to the treatment [118].

Despite promising results, studies mentioned above have certain limitations. The patients included have been diagnosed with NETs of different primary locations (foregut, midgut and hindgut tumors), therefore their biological features might differ. Moreover, different treatment methods (SSA included) might have affected the CTC expression and, therefore, the results [119]. The biomarker issue aside, recent findings suggest that a qualitative and quantitative assessment of CTC may be equally important. Mutations present in CTC reflect the genomic aberrations found in tumor tissue, making liquid biopsy a useful option in cases where standard biopsy might not be possible or for tracking changes in a tumor's genomic landscape. Monitoring these changes can also be useful in establishing mechanisms of resistance to certain forms of treatment [120].

NETs are generally indolent tumors; about a fifth of the patients present with metastases at diagnosis [121]. In mouse models of aggressive tumors, such as breast or pancreatic cancer, CTC have been detected even at the early stage of the disease [122]. However, the question remains whether the same can be applied to NETs given CTC's limited sensitivity in tumors with more metastatic potential. What is more, CTC's potential uses as prognostic or predictive biomarkers require further research. With the lack of a large cohort, multicenter studies remain an important unmet need.

3.8. Circulating Tumor DNA and Cell-Free DNA

Circulating tumor DNA (ctDNA) and cell-free DNA (cfDNA) are a novel tool that can be used to describe NETs molecular features. Whereas ctDNA are fragments of DNA derived from a tumor and found in the circulation, cfDNA are a broader term and also include fragments of nucleic acid that do not originate in a tumor. The main source of circulating tumor DNA seems to be the apoptosis, although the exact mechanism of releasing ctDNA into the body fluids remains unclear [123]. The principle behind this test is the identification of circulating DNA and its molecular rearrangements, which may affect treatment choices [124].

The presence of ctDNA was first reported in 1948 by Mandel and Metais, who detected cell-free nucleic acids in the blood of cancer patients [125]. Since then, ctDNA has been widely studied as an alternative for tissue biopsies in malignancies, however, the data about its use in NETs remains scarce. A relative lack of known, unique to NEN, neoplasm-promoting mutations presents a significant limitation for the use of ctDNA [126,127].

Some of the upsides of circulating nucleic acid analysis include the simplicity of obtaining the material and minimally invasive monitoring of the tumor during therapy by liquid biopsy. The risk of false negative results seems to be the main limitation of this method, due to variable amounts of DNA that tumors may release into circulation [124,128].

It has been reported that the presence of ctDNA in body fluids is linked to the localization of the primary tumor and metastatic lesions [129–132]. Oversoe et al. described elevated levels of cfDNA in panNET and siNET patients compared to healthy controls [133]. Tumors with liver metastases and a high proliferative index and necrosis, features which are often characteristic of NEC, are associated with a high ctDNA concentration [131]. Boons et al., described a correlation between the presence of ctDNA and a higher grading [132]. On the contrary to NECs, NETs (which are generally slow growing tumors) have a lower cell loss index and ctDNA release and can often be ctDNA negative [130]. Quantitative analysis of ctDNA may also be useful to assess tumor volume and in an early diagnosis of relapse after surgery and as a predictive factor of response to treat-

ment [134–137]. OS and PFS appear to be significantly worse in ctDNA-positive than in ctDNA-negative patients [130].

Another important aspect of cfDNA analysis is the possibility of methylation pattern analysis. Abnormal distribution of DNA methylation has been described in early carcinogenesis and may be helpful in the detection, monitoring and treatment response prediction [138]. A number of studies have been performed describing in NET tumor tissue methylation patterns compared to healthy controls [139–142]. Mettler et al. analyzed the cfDNA characteristics of 63 NEN patients in comparison with healthy controls. A higher cfDNA concentration and hypomethylation patterns have been found in advanced NEN and their association with tumor burden and a worse prognosis has been described [143].

Although the sensitivity of ctDNA may be lower than the currently used analytes, it is a highly specific biomarker, which can be especially useful in rare diseases [130,144]. Moreover, in the qualitative analysis of ctDNA, both copy number alterations and point mutations in DNA is clinically relevant, namely for screening patients who are eligible for targeted therapies. However, the application of ctDNA in NETs requires further study [145].

3.9. Other Potential Biomarkers and 19th Annual Enets Conference Abstracts

Some other areas of interest in the field of NET biomarkers have been described in recent years that are not covered in detail by this review, e.g., long non-coding RNA and tumor-infiltrating platelets. However, the data on these remains scarce [146,147]. In a recent report, Hinterleitner et al. described elevated levels of platelet-expressed synaptophysin (pSyn) in NEN compared to healthy donors. A high expression of pSyn was shown to correlate with a shorter PFS, higher tumor stages, the presence of metastases and a higher tumor proliferation rate [148].

The 19th Annual ENETS Conference took place in March 2022. Some of the research presented during the conference focused on NEN biomarker development. La Salvia et al. presented an analysis of extra-pancreatic NETs metabolomics profile, some of which can be used as independent prognostic biomarkers. Some of the findings have already been published in a peer-reviewed journal [149,150]. Another interesting finding has been the analysis of Copy Number Alterations (CNAs) in cfDNA. The method utilizes whole-genome sequencing of cfDNA (its ctDNA fraction, precisely) in material acquired by liquid biopsy. CNAs found in analyzed material showed a sensitivity and specificity for NENs of 62% and 86%, respectively [151]. Garcia Alvarez et al. analyzed the plasma of panNETs and giNETs prior to the start of Lenvatinib. High levels of angiopoietin 2 (Ang2) and low levels of fibroblast growth factor 2 (FGF-2) resulted in a better response to treatment, which may point to them being useful as predictive biomarkers [152]. One study focused on ctDNA in NEN and its clinical utility for monitoring. The lack of identifiable ctDNA in patients with stable disease has been described, which may help in selecting a group of patients with no need for intensive monitoring [153]. Serum Activin A has been researched as an alternative to NT-proBNP in CHD patients, however, its diagnostic parameters for the detection of CHD have been subpar [154]. Schalin-Jantti et al. presented an analysis of clinical factors (CF) and novel plasma proteins (NPP) in G1 and G2 SI-NET patients using data mining and machine learning methods. The study focused on establishing a multi biomarker strategy for NET. The combination of CF and NPP allowed for the identification of stable and progressive disease subgroups [155]. This research is yet another example of how useful machine learning might be in advancing patient care. Finally, a study focusing on an NETest have been presented by van Treijen et al. showing its function in predicting treatment response and individualizing treatment decision [156]. The latter conclusion is especially important as the individualization of therapy has been a major talking point during the 19th Annual ENETS Conference.

4. Conclusions

Over the years, multiple NET biomarkers have been researched, developed and used. From simple substances secreted by the tumor to complex mathematical algorithms,

there is a wide range of biomarkers to choose from. Despite this, there is still an unmet need for the development of widely available and accurate NET biomarkers. Experts specializing in NETs agree that the currently used analytes have several limitations and that multianalyte panels based on the genetic signature of the tumor should be the course of future research [49]. Describing different aspects of a disease as complex and heterogeneous as NET based on a single substance is insufficient. In comparison, utilizing mathematical algorithms allows for a more comprehensive depiction of the state of the disease (thanks to the numerous variables that are included, instead of just a single one) [157]. In a recent study, Kidd et al. described the potential expansion of the NETest, improving its statistical parameters even further [158]. This is yet another advantage of machine learning algorithms; With new discoveries, they can be tweaked for even more accurate analysis. A question often raised is the cost effectiveness of the new biomarkers [159]. Measuring a single substance is markedly less expensive than molecular tests, however, a more efficient biomarker will allow for a decreased spending on imaging and treatment [80].

As shown by this review, there is still room for improvement in the field of NET biomarkers. A number of analytes, such as miRNA, CTC and NETest have shown promising results, however, their use in daily clinical practice is currently limited by either their low availability or lack of standardization.

Out of the potential biomarkers mentioned above, the NETest offers superior diagnostic parameters compared to traditional analytes and has been shown to detect progression and disease recurrence quicker than imaging methods. It is also useful in the assessment of the response to radioisotope treatment and radicality of surgical intervention. As stated in the recently published guidelines of the Polish Network of Neuroendocrine Tumours, the use of an NETest in everyday clinical practice will enable the optimal inclusion of the test in the management algorithms in the Polish population of patients with NEN [8].

However, with the NETest limited availability, there is still place for traditional analytes. In accordance with the updated guidelines of the Polish Network of Neuroendocrine Tumours, we advise utilizing CgA for monitoring during treatment and as a prognostic biomarker in colorectal NEN [8,160]. In small intestine and pancreatic NEN, measuring CgA has a utility before introducing treatment and for monitoring. Additionally, in patients diagnosed with small intestine NEN, bronchopulmonary NEN or when suspecting carcinoid syndrome, it is recommended to measure 5-HIAA in urine (at least two samples, collected over 24 h period each) [8,161,162]. Though not a NET biomarker sensu stricto, the N-terminal prohormone of brain natriuretic peptide (NT-proBNP) should be measured for the diagnosis and monitoring of carcinoid heart disease in carcinoid syndrome patients [163]. In patients presenting with symptoms characteristic of functional NETs, we recommend measuring the hormones linked with the suspected syndrome (as shown on Table 2). As discussed earlier in this review, medical decisions shouldn't be taken solely on the basis of change in biomarker concentration, due to their several limitations.

To summarize, despite the recent advances in the field of NET biomarkers, novel analytes have not yet been introduced into wider use. Some of them (such as an NETest) show a lot of promise and with a wider availability, they offer a significant improvement over traditional analytes. Until they become a routine tool in NET diagnostics, biomarkers such as CgA and 5-HIAA can still be a helpful option in select cases.

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References

1. Das, S.; Dasari, A. Epidemiology, Incidence, and Prevalence of Neuroendocrine Neoplasms: Are There Global Differences? *Curr. Oncol. Rep.* **2021**, *23*, 43. [CrossRef]
2. Dasari, A.; Shen, C.; Halperin, D.; Zhao, B.; Zhou, S.; Xu, Y.; Shih, T.; Yao, J.C. Trends in the Incidence, Prevalence, and Survival Outcomes in Patients with Neuroendocrine Tumors in the United States. *JAMA Oncol.* **2017**, *3*, 1335–1342. [CrossRef]
3. Taal, B.G.; Visser, O. Epidemiology of neuroendocrine tumours. *Neuroendocrinology* **2004**, *80* (Suppl. S1), 3–7. [CrossRef]
4. Assarzadegan, N.; Montgomery, E. What is New in the 2019 World Health Organization (WHO) Classification of Tumors of the Digestive System: Review of Selected Updates on Neuroendocrine Neoplasms, Appendiceal Tumors, and Molecular Testing. *Arch. Pathol. Lab. Med.* **2021**, *145*, 664–677. [CrossRef]
5. Rindi, G.; Mete, O.; Uccella, S.; Basturk, O.; La Rosa, S.; Brosens, L.; Ezzat, S.; de Herder, W.W.; Klimstra, D.S.; Papotti, M.; et al. Overview of the 2022 WHO Classification of Neuroendocrine Neoplasms. *Endocr. Pathol.* **2022**, *33*, 115–154. [CrossRef]
6. Williams, E.D.; Sandler, M. The classification of carcinoid tumors. *Lancet.* **1963**, *281*, 238–239. [CrossRef]
7. Al-Khafaji, B.; Noffsinger, A.E.; Miller, M.A.; DeVoe, G.; Stemmermann, G.N.; Fenoglio-Preiser, C. Immunohistologic analysis of gastrointestinal and pulmonary carcinoid tumors. *Hum. Pathol.* **1998**, *29*, 992–999. [CrossRef]
8. Kos-Kudła, B.; Foltyn, W.; Malczewska, A.; Bednarczuk, T.; Bolanowski, M.; Borowska, M.; Chmielik, E.; Ćwikła, J.B.; Gisterek, I.; Handkiewicz-Junak, D.; et al. Update of the diagnostic and therapeutic guidelines for gastro-entero-pancreatic neuroendocrine neoplasms (recommended by the Polish Network of Neuroendocrine Tumours). *Endokrynol. Pol.* **2022**, *73*, 387–454. [CrossRef]
9. Falconi, M.; Eriksson, B.; Kaltsas, G.; Bartsch, D.K.; Capdevila, J.; Caplin, M.; Kos-Kudla, B.; Kwekkeboom, D.; Rindi, G.; Klöppel, G.; et al. ENETS Consensus Guidelines Update for the Management of Patients with Functional Pancreatic Neuroendocrine Tumors and Non-Functional Pancreatic Neuroendocrine Tumors. *Neuroendocrinology* **2016**, *103*, 153–171. [CrossRef]
10. Okabayashi, T.; Shima, Y.; Sumiyoshi, T.; Kozuki, A.; Ito, S.; Ogawa, Y.; Kobayashi, M.; Hanazaki, K. Diagnosis and management of insulinoma. *World J. Gastroenterol.* **2013**, *19*, 829–837. [CrossRef]
11. Shah, M.H.; Goldner, W.S.; Benson, A.B.; Bergsland, E.; Blaszkowsky, L.S.; Brock, P.; Chan, J.; Satya, D.; Dickson, P.; Fanta, P.; et al. Neuroendocrine and Adrenal Tumors, Version 1.2022, NCCN Clinical Practice Guidelines in Oncology. Available online: https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1448 (accessed on 29 August 2022).
12. Blaschko, H.; Comline, R.S.; Schneider, F.H.; Silver, M.; Smith, A.D. Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. *Nature* **1967**, *215*, 58–59. [CrossRef] [PubMed]
13. Feldman, S.A.; Eiden, L.E. The chromogranins: Their roles in secretion from neuroendocrine cells and as markers for neuroendocrine neoplasia. *Endocr. Pathol.* **2003**, *14*, 3–23. [CrossRef]
14. Winkler, H.; Fischer-Colbrie, R. The chromogranins A and B: The first 25 years and future perspectives. *Neuroscience* **1992**, *49*, 497–528. [CrossRef]
15. Huttner, W.B.; Gerdes, H.H.; Rosa, P. The granin (chromogranin/secretogranin) family. *Trends Biochem. Sci.* **1991**, *16*, 27–30. [CrossRef]
16. Montero-Hadjadje, M.; Vaingankar, S.; Elias, S.; Tostivint, H.; Mahata, S.K.; Anouar, Y. Chromogranins A and B and secretogranin II: Evolutionary and functional aspects. *Acta Physiol.* **2008**, *192*, 309–324. [CrossRef]
17. Bartolomucci, A.; Possenti, R.; Mahata, S.K.; Fischer-Colbrie, R.; Loh, Y.P.; Salton, S.R. The extended granin family: Structure, function, and biomedical implications. *Endocr. Rev.* **2011**, *32*, 755–797. [CrossRef]
18. Gould, V.E. Neuroendocrinomas and neuroendocrine carcinomas: APUD cell system neoplasms and their aberrant secretory activities. *Pathol. Annu.* **1977**, *12*, 33–62.
19. Hsiao, R.J.; Parmer, R.J.; Takiyyuddin, M.A.; O'Connor, D.T. Chromogranin A storage and secretion: Sensitivity and specificity for the diagnosis of pheochromocytoma. *Medicine* **1991**, *70*, 33–45. [CrossRef]
20. Deftos, L.J. Chromogranin A: Its role in endocrine function and as an endocrine and neuroendocrine tumor marker. *Endocr. Rev.* **1991**, *12*, 181–187. [CrossRef]
21. Kanakis, G.; Kaltsas, G. Biochemical markers for gastroenteropancreatic neuroendocrine tumours (GEP-NETs). *Best Pract. Res. Clin. Gastroenterol.* **2012**, *26*, 791–802. [CrossRef]
22. Oberg, K.; Couvelard, A.; Delle Fave, G.; Gross, D.; Grossmann, A.; Jensen, R.T.; Pape, U.F.; Perren, A.; Rindi, G.; Ruszniewski, P.; et al. ENETS Consensus Guidelines for Standard of Care in Neuroendocrine Tumours: Biochemical Markers. *Neuroendocrinology* **2017**, *105*, 201–211. [CrossRef] [PubMed]
23. Halfdanarson, T.R.; Strosberg, J.R.; Tang, L.; Bellizzi, A.M.; Bergsland, E.K.; O'Dorisio, T.M.; Halperin, D.M.; Fishbein, L.; Eads, J.; Hope, T.A.; et al. The North American Neuroendocrine Tumor Society Consensus Guidelines for Surveillance and Medical Management of Pancreatic Neuroendocrine Tumors. *Pancreas* **2020**, *49*, 863–881. [CrossRef] [PubMed]

24. Arnold, R.; Wilke, A.; Rinke, A.; Mayer, C.; Kann, P.H.; Klose, K.J.; Scherag, A.; Hahmann, M.; Müller, H.H.; Barth, P. Plasma chromogranin A as marker for survival in patients with metastatic endocrine gastroenteropancreatic tumors. *Clin. Gastroenterol. Hepatol.* **2008**, *6*, 820–827. [CrossRef] [PubMed]

25. Bajetta, E.; Ferrari, L.; Martinetti, A.; Celio, L.; Procopio, G.; Artale, S.; Zilembo, N.; Di Bartolomeo, M.; Seregni, E.; Bombardieri, E. Chromogranin A, neuron specific enolase, carcinoembryonic antigen, and hydroxyindole acetic acid evaluation in patients with neuroendocrine tumors. *Cancer* **1999**, *86*, 858–865. [CrossRef]

26. Yang, X.; Yang, Y.; Li, Z.; Cheng, C.; Yang, T.; Wang, C.; Liu, L.; Liu, S. Diagnostic value of circulating chromogranin a for neuroendocrine tumors: A systematic review and meta-analysis. *PLoS ONE* **2015**, *10*, e0124884. [CrossRef] [PubMed]

27. O'Connor, D.T.; Deftos, L.J. Secretion of chromogranin A by peptide-producing endocrine neoplasms. *N. Engl. J. Med.* **1986**, *314*, 1145–1151. [CrossRef]

28. Nobels, F.R.; Kwekkeboom, D.J.; Coopmans, W.; Schoenmakers, C.H.; Lindemans, J.; De Herder, W.W.; Krenning, E.P.; Bouillon, R.; Lamberts, S.W. Chromogranin A as serum marker for neuroendocrine neoplasia: Comparison with neuron-specific enolase and the alpha-subunit of glycoprotein hormones. *J. Clin. Endocrinol. Metab.* **1997**, *82*, 2622–2628. [CrossRef]

29. Rydzewska, G.; Strzelczyk, J.; . Bednarczuk, T.; Bolanowski, M.; Borowska, M.; Chmielik, E.; Ćwikła, J.B.; Foltyn, W.; Gisterek, I.; Handkiewicz-Junak, D.; et al. Gastroduodenal neuroendocrine neoplasms including gastrinoma—update of the diagnostic and therapeutic guidelines (recommended by the Polish Network of Neuroendocrine Tumours). *Endokrynol. Pol.* **2022**, *73*, 455–490. [CrossRef]

30. Rossi, R.E.; Ciafardini, C.; Sciola, V.; Conte, D.; Massironi, S. Chromogranin A in the Follow-up of Gastroenteropancreatic Neuroendocrine Neoplasms: Is It Really Game Over? A Systematic Review and Meta-analysis. *Pancreas* **2018**, *47*, 1249–1255. [CrossRef]

31. Rossi, R.E.; Garcia-Hernandez, J.; Meyer, T.; Thirlwell, C.; Watkins, J.; Martin, N.G.; Caplin, M.E.; Toumpanakis, C. Chromogranin A as a predictor of radiological disease progression in neuroendocrine tumours. *Ann. Transl. Med.* **2015**, *3*, 118. [CrossRef]

32. Massironi, S.; Rossi, R.E.; Casazza, G.; Conte, D.; Ciafardini, C.; Galeazzi, M.; Peracchi, M. Chromogranin A in diagnosing and monitoring patients with gastroenteropancreatic neuroendocrine neoplasms: A large series from a single institution. *Neuroendocrinology* **2014**, *100*, 240–249. [CrossRef] [PubMed]

33. Malczewska, A.; Kidd, M.; Matar, S.; Kos-Kudła, B.; Bodei, L.; Oberg, K.; Modlin, I.M. An Assessment of Circulating Chromogranin A as a Biomarker of Bronchopulmonary Neuroendocrine Neoplasia: A Systematic Review and Meta-Analysis. *Neuroendocrinology* **2020**, *110*, 198–216. [CrossRef] [PubMed]

34. Matar, S.; Malczewska, A.; Oberg, K.; Bodei, L.; Aslanian, H.; Lewczuk-Myślicka, A.; Filosso, P.L.; Suarez, A.L.; Kolasińska-Ćwikła, A.; Roffinella, M.; et al. Blood Chromogranin A Is Not Effective as a Biomarker for Diagnosis or Management of Bronchopulmonary Neuroendocrine Tumors/Neoplasms. *Neuroendocrinology* **2020**, *110*, 185–197. [CrossRef] [PubMed]

35. Caplin, M.E.; Baudin, E.; Ferolla, P.; Filosso, P.; Garcia-Luste, M.; Lim, E.; Oberg, K.; Pelosi, G.; Perren, A.; Rossi, R.E. Pulmonary neuroendocrine (carcinoid) tumors: European Neuroendocrine Tumor Society expert consensus and recommendations for best practice for typical and atypical pulmonary carcinoids. *Ann. Oncol.* **2015**, *26*, 1604–1620. [CrossRef] [PubMed]

36. Glinicki, P.; Kapuścińska, R.; Jeske, W. The differences in chromogranin A (CgA) concentrations measured in serum and in plasma by IRMA and ELISA methods. *Endokrynol. Pol.* **2010**, *61*, 346–350.

37. Glinicki, P.; Jeske, W. Chromogranina A (CgA)—wpływ różnych czynników in vivo, in vitro i istniejących chorób na jej stężenia we krwi [Chromogranin A (CgA)—the influence of various factors in vivo and in vitro, and existing disorders on it's concentration in blood]. *Endokrynol. Pol.* **2011**, *62* (Suppl. S1), 25–28.

38. Sciola, V.; Massironi, S.; Conte, D.; Caprioli, F.; Ferrero, S.; Ciafardini, C.; Peracchi, M.; Bardella, M.T.; Piodi, L. Plasma chromogranin a in patients with inflammatory bowel disease. *Inflamm. Bowel Dis.* **2009**, *15*, 867–871. [CrossRef]

39. Pregun, I.; Herszényi, L.; Juhász, M.; Miheller, P.; Hritz, I.; Patocs, A.; Racz, K.; Tulassay, Z. Effect of proton-pump inhibitor therapy on serum chromogranin a level. *Digestion* **2011**, *84*, 22–28. [CrossRef]

40. Lanzon-Miller, S.; Pounder, R.E.; Hamilton, M.R.; Ball, S.; Raymond, F.; Olausson, M.; Cederberg, C. Twenty-four-hour intragastric acidity and plasma gastrin concentration before and during treatment with either ranitidine or omeprazole. *Aliment. Pharmacol. Ther.* **1987**, *1*, 239–251. [CrossRef]

41. Waldum, H.L.; Syversen, U. Serum chromogranin A in the control of patients on long-term treatment with inhibitors of acid secretion. *Eur. J. Clin. Investig.* **2001**, *31*, 741–743. [CrossRef]

42. Korse, C.M.; Muller, M.; Taal, B.G. Discontinuation of proton pump inhibitors during assessment of chromogranin A levels in patients with neuroendocrine tumours. *Br. J. Cancer* **2011**, *105*, 1173–1175. [CrossRef] [PubMed]

43. Gut, P.; Czarnywojtek, A.; Fischbach, J.; Bączyk, M.; Ziemnicka, K.; Wrotkowska, E.; Gryczyńska, M.; Ruchała, M. Chromogranin A—unspecific neuroendocrine marker. Clinical utility and potential diagnostic pitfalls. *Arch. Med. Sci.* **2016**, *12*, 1–9. [CrossRef]

44. Raines, D.; Chester, M.; Diebold, A.E.; Mamikunian, P.; Anthony, C.T.; Mamikunian, G.; Woltering, E.A. A prospective evaluation of the effect of chronic proton pump inhibitor use on plasma biomarker levels in humans. *Pancreas* **2012**, *41*, 508–511. [CrossRef] [PubMed]

45. Ito, T.; Igarashi, H.; Jensen, R.T. Serum pancreastatin: The long sought universal, sensitive, specific tumor marker for neuroendocrine tumors? *Pancreas* **2012**, *41*, 505–507. [CrossRef] [PubMed]

46. Sherman, S.K.; Maxwell, J.E.; O'Dorisio, M.S.; O'Dorisio, T.M.; Howe, J.R. Pancreastatin predicts survival in neuroendocrine tumors. *Ann. Surg. Oncol.* **2014**, *21*, 2971–2980. [CrossRef]

47. Khan, T.M.; Garg, M.; Warner, R.R.; Uhr, J.H.; Divino, C.M. Elevated Serum Pancreastatin Is an Indicator of Hepatic Metastasis in Patients with Small Bowel Neuroendocrine Tumors. *Pancreas* **2016**, *45*, 1032–1035. [CrossRef]
48. Tran, C.G.; Sherman, S.K.; Scott, A.T.; Ear, P.H.; Chandrasekharan, C.; Bellizzi, A.M.; Dillon, J.S.; O'Dorisio, T.M.; Howe, J.R. It Is Time to Rethink Biomarkers for Surveillance of Small Bowel Neuroendocrine Tumors. *Ann. Surg. Oncol.* **2021**, *28*, 732–741. [CrossRef]
49. Oberg, K.; Modlin, I.M.; De Herder, W.; Pavel, M.; Klimstra, D.; Frilling, A.; Metz, D.C.; Heaney, A.; Kwekkeboom, D.; Strosberg, J.; et al. Consensus on biomarkers for neuroendocrine tumour disease. *Lancet Oncol.* **2015**, *16*, 435–446. [CrossRef]
50. Bellono, N.W.; Bayrer, J.R.; Leitch, D.B.; Castro, J.; Zhang, C.; O'Donnell, T.A.; Brierley, S.M.; Ingraham, H.A.; Julius, D. Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. *Cell* **2017**, *170*, 185–198. [CrossRef]
51. Tsoucalas, G.; Karamanou, M.; Androutsos, G. The eminent German pathologist Siegfried Oberndorfer (1876–1944) and his landmark work on carcinoid tumors. *Ann. Gastroenterol.* **2011**, *24*, 98–100.
52. Sansone, A.; Lauretta, R.; Vottari, S.; Chiefari, A.; Barnabei, A.; Romanelli, F.; Appetecchia, M. Specific and Non-Specific Biomarkers in Neuroendocrine Gastroenteropancreatic Tumors. *Cancers* **2019**, *11*, 1113. [CrossRef] [PubMed]
53. Deacon, A.C. The measurement of 5-hydroxyindoleacetic acid in urine. *Ann. Clin. Biochem* **1994**, *31*, 215–232. [CrossRef]
54. Aluri, V.; Dillon, J.S. Biochemical Testing in Neuroendocrine Tumors. *Endocrinol. Metab. Clin. N. Am.* **2017**, *46*, 669–677. [CrossRef] [PubMed]
55. Formica, V.; Wotherspoon, A.; Cunningham, D.; Norman, A.R.; Sirohi, B.; Oates, J.; Chong, G. The prognostic role of WHO classification, urinary 5-hydroxyindoleacetic acid and liver function tests in metastatic neuroendocrine carcinomas of the gastroenteropancreatic tract. *Br. J. Cancer* **2007**, *96*, 1178–1182. [CrossRef] [PubMed]
56. Niederle, B.; Pape, U.F.; Costa, F.; Gross, D.; Kelestimur, F.; Knigge, U.; Öberg, K.; Pavel, M.; Perren, A.; Toumpanakis, C.; et al. ENETS Consensus Guidelines Update for Neuroendocrine Neoplasms of the Jejunum and Ileum. *Neuroendocrinology* **2016**, *103*, 125–138. [CrossRef]
57. Holzer, P.; Reichmann, F.; Farzi, A. Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. *Neuropeptides* **2012**, *46*, 261–274. [CrossRef]
58. Walter, T.; Chardon, L.; Chopin-laly, X.; Raverot, V.; Caffin, A.G.; Chayvialle, J.A.; Scoazec, J.Y.; Lombard-Bohas, C. Is the combination of chromogranin A and pancreatic polypeptide serum determinations of interest in the diagnosis and follow-up of gastro-entero-pancreatic neuroendocrine tumours? *Eur. J. Cancer* **2012**, *48*, 1766–1773. [CrossRef]
59. Panzuto, F.; Severi, C.; Cannizzaro, R.; Falconi, M.; Angeletti, S.; Pasquali, A.; Corleto, V.D.; Annibale, B.; Buonadonna, A.; Pederzoli, P.; et al. Utility of combined use of plasma levels of chromogranin A and pancreatic polypeptide in the diagnosis of gastrointestinal and pancreatic endocrine tumors. *J. Endocrinol. Investig.* **2004**, *27*, 6–11. [CrossRef]
60. Iwafuchi, M.; Watanabe, H.; Ishihara, N.; Shimoda, T.; Iwashita, A.; Ito, S. Peptide YY immunoreactive cells in gastrointestinal carcinoids: Immunohistochemical and ultrastructural studies of 60 tumors. *Hum. Pathol.* **1986**, *17*, 291–296. [CrossRef]
61. Kim, J.Y.; Kim, K.S.; Kim, K.J.; Park, I.J.; Lee, J.L.; Myung, S.J.; Park, Y.; Park, Y.S.; Yu, C.S.; Kim, J.C.; et al. Non-L-cell immunophenotype and large tumor size in rectal neuroendocrine tumors are associated with aggressive clinical behavior and worse prognosis. *Am. J. Surg. Pathol.* **2015**, *39*, 632–643. [CrossRef]
62. Grouzmann, E.; Comoy, E.; Bohuon, C. Plasma neuropeptide Y concentrations in patients with neuroendocrine tumors. *J. Clin. Endocrin. Metab.* **1989**, *68*, 808–813. [CrossRef] [PubMed]
63. Plouin, P.F.; Bertherat, J.; Chatellier, G.; Billaud, E.; Azizi, M.; Grouzmann, E.; Epelbaum, J. Short-term effects of octreotide on blood pressure and plasma catecholamines and neuropeptide Y levels in patients with phaeochromocytoma: A placebo-controlled trial. *Clin. Endocrinol.* **1995**, *42*, 289–294. [CrossRef] [PubMed]
64. Allen, J.M.; Yeats, J.C.; Causon, R.; Brown, M.J.; Bloom, S.R. Neuropeptide Y and its flanking peptide in human endocrine tumors and plasma. *J. Clin. Endocrinol. Metab.* **1987**, *64*, 1199–1204. [CrossRef] [PubMed]
65. Moore, B.W.; McGregor, D. Chromatographic and Electrophoretic Fractionation of Soluble Proteins of Brain and Liver. *J. Biol. Chem.* **1965**, *240*, 1647–1653. [CrossRef]
66. Isgrò, M.A.; Bottoni, P.; Scatena, R. Neuron-Specific Enolase as a Biomarker: Biochemical and Clinical Aspects. *Adv. Exp. Med. Biol.* **2015**, *867*, 125–143. [CrossRef]
67. Gut, P.; Czarnywojtek, A.; Sawicka-Gutaj, N.; Woliński, K.; Maciejewski, A.; Komarnicki, P.; Ruchała, M. Determination of neuron-specific enolase in patients with midgut-type tumour treated with somatostatin analogues. *Endokrynol. Pol.* **2021**, *72*, 308–318. [CrossRef]
68. Korse, C.M.; Taal, B.G.; Vincent, A.; van Velthuysen, M.-L.F.; Baas, P.; Buning-Kager, J.C.G.M.; Linders, T.C.; Bongrer, J.M.G. Choice of tumour markers in patients with neuroendocrine tumours is dependent on the histological grade. A marker study of Chromogranin A, Neuron specific enolase, Progastrin-releasing peptide and cytokeratin fragments. *Eur. J. Cancer* **2012**, *48*, 662–671. [CrossRef]
69. Kečkéš, Š.; Palaj, J.; Waczulíková, I.; Dyttert, D.; Mojtová, E.; Kováč, G.; Durdík, Š. Pretreatment Levels of Chromogranin A and Neuron-specific Enolase in Patients With Gastroenteropancreatic Neuroendocrine Neoplasia. *In Vivo* **2021**, *35*, 2863–2868. [CrossRef]
70. Modlin, I.M.; Moss, S.F.; Chung, D.C.; Jensen, R.T.; Snyderwine, E. Priorities for improving the management of gastroenteropancreatic neuroendocrine tumors. *J. Natl. Cancer Inst.* **2008**, *100*, 1282–1289. [CrossRef]

71. Kidd, M.; Modlin, I.M.; Bodei, L.; Drozdov, I. Decoding the Molecular and Mutational Ambiguities of Gastroenteropancreatic Neuroendocrine Neoplasm Pathobiology. *Cell. Mol. Gastroenterol. Hepatol.* **2015**, *1*, 131–153. [CrossRef]

72. Modlin, I.M.; Kidd, M.; Malczewska, A.; Drozdov, I.; Bodei, L.; Matar, S.; Chung, K.M. The NETest: The Clinical Utility of Multigene Blood Analysis in the Diagnosis and Management of Neuroendocrine Tumors. *Endocrinol. Metab. Clin. N. Am.* **2018**, *47*, 485–504. [CrossRef]

73. Al-Toubah, T.; Cives, M.; Valone, T.; Blue, K.; Strosberg, J. Sensitivity and Specificity of the NETest: A Validation Study. *Neuroendocrinology* **2021**, *111*, 580–585. [CrossRef] [PubMed]

74. Modlin, I.M.; Drozdov, I.; Kidd, M. The identification of gut neuroendocrine tumor disease by multiple synchronous transcript analysis in blood. *PLoS ONE* **2013**, *8*, e63364. [CrossRef] [PubMed]

75. Modlin, I.M.; Aslanian, H.; Bodei, L.; Drozdov, I.; Kidd, M. A PCR blood test outperforms chromogranin A in carcinoid detection and is unaffected by proton pump inhibitors. *Endocr. Connect.* **2014**, *3*, 215–223. [CrossRef]

76. Van Treijen, M.J.C.; Korse, C.M.; van Leeuwaarde, R.S.; Saveur, L.J.; Vriens, M.R.; Verbeek, W.H.M.; Tesselaar, M.E.T.; Valk, G.D. Blood Transcript Profiling for the Detection of Neuroendocrine Tumors: Results of a Large Independent Validation Study. *Front. Endocrinol.* **2018**, *9*, 740. [CrossRef] [PubMed]

77. Modlin, I.M.; Kidd, M.; Falconi, M.; Filosso, P.L.; Frilling, A.; Malczewska, A.; Toumpanakis, C.; Valk, G.; Pacak, K.; Bodei, L. A multigenomic liquid biopsy biomarker for neuroendocrine tumor disease outperforms CgA and has surgical and clinical utility. *Ann. Oncol.* **2021**, *32*, 1425–1433. [CrossRef] [PubMed]

78. Pavel, M.; Jann, H.; Prasad, V.; Drozdov, I.; Modlin, I.M.; Kidd, M. NET Blood Transcript Analysis Defines the Crossing of the Clinical Rubicon: When Stable Disease Becomes Progressive. *Neuroendocrinology* **2017**, *104*, 170–182. [CrossRef]

79. Malczewska, A.; Kos-Kudła, B.; Kidd, M.; Drozdov, I.; Bodei, L.; Matar, S.; Oberg, K.; Modlin, T.M. The clinical applications of a multigene liquid biopsy (NETest) in neuroendocrine tumors. *Adv. Med. Sci.* **2020**, *65*, 18–29. [CrossRef]

80. Öberg, K.; Califano, A.; Strosberg, J.R.; Ma, S.; Pape, U.; Bodei, L.; Kaltsas, G.; Toumpanakis, C.; Goldenring, J.R.; Frilling, A.; et al. A meta-analysis of the accuracy of a neuroendocrine tumor mRNA genomic biomarker (NETest) in blood. *Ann. Oncol.* **2020**, *31*, 202–212. [CrossRef]

81. Malczewska, A.; Witkowska, M.; Wójcik-Giertuga, M.; Kuśnierz, K.; Bocian, A.; Walter, A.; Rydel, M.; Robek, A.; Pierzchała, S.; Malczewska, M.; et al. Prospective Evaluation of the NETest as a Liquid Biopsy for Gastroenteropancreatic and Bronchopulmonary Neuroendocrine Tumors: An ENETS Center of Excellence Experience. *Neuroendocrinology* **2021**, *111*, 304–319. [CrossRef]

82. Liu, E.; Paulson, S.; Gulati, A.; Freudman, J.; Grosh, W.; Kafer, S.; Wickremesinghe, P.C.; Salem, R.R.; Bodei, L. Assessment of NETest Clinical Utility in a U.S. Registry-Based Study. *Oncologist* **2019**, *24*, 783–790. [CrossRef] [PubMed]

83. Kidd, M.; Drozdov, I.A.; Matar, S.; Gurunlian, N.; Ferranti, N.J.; Malczewska, A.; Bennett, P.; Bodei, L.; Modlin, I.M. Utility of a ready-to-use PCR system for neuroendocrine tumor diagnosis. *PLoS ONE* **2019**, *14*, e0218592. [CrossRef] [PubMed]

84. Lu, T.X.; Rothenberg, M.E. MicroRNA. *J. Allergy Clin. Immunol.* **2018**, *141*, 1202–1207. [CrossRef]

85. Lee, R.C.; Feinbaum, R.L.; Ambros, V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **1993**, *75*, 843–854. [CrossRef]

86. O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol.* **2018**, *9*, 402. [CrossRef] [PubMed]

87. Kai, K.; Dittmar, R.L.; Sen, S. Secretory microRNAs as biomarkers of cancer. *Semin. Cell Dev. Biol.* **2018**, *78*, 22–36. [CrossRef] [PubMed]

88. Zhang, J.; Zhao, H.; Gao, Y.; Zhang, W. Secretory miRNAs as novel cancer biomarkers. *Biochim. Biophys. Acta* **2012**, *1826*, 32–43. [CrossRef]

89. Paul, P.; Chakraborty, A.; Sarkar, D.; Langthasa, M.; Rahman, M.; Bari, M.; Singha, R.S.; Malakar, A.K.; Chakraborty, S. Interplay between miRNAs and human diseases. *J. Cell. Physiol.* **2018**, *233*, 2007–2018. [CrossRef]

90. van den Berg, M.M.J.; Krauskopf, J.; Ramaekers, J.G.; Kleinjans, J.C.S.; Prickaerts, J.; Briedé, J.J. Circulating microRNAs as potential biomarkers for psychiatric and neurodegenerative disorders. *Prog. Neurobiol.* **2020**, *185*, 101732. [CrossRef]

91. Croce, C.M. Causes and consequences of microRNA dysregulation in cancer. *Nat. Rev. Genet.* **2009**, *10*, 704–714. [CrossRef]

92. Zhao, L.; Liang, X.; Wang, L.; Zhang, X. The Role of miRNA in Ovarian Cancer: An Overview. *Reprod. Sci.* **2022**. [CrossRef]

93. Lampignano, R.; Kloten, V.; Krahn, T.; Schlange, T. Integrating circulating miRNA analysis in the clinical management of lung cancer: Present or future? *Mol. Aspects Med.* **2020**, *72*, 100844. [CrossRef] [PubMed]

94. Toiyama, Y.; Okugawa, Y.; Fleshman, J.; Boland, C.R.; Goel, A. MicroRNAs as potential liquid biopsy biomarkers in colorectal cancer: A systematic review. *Biochim. Biophys. Acta Rev. Cancer* **2018**, *1870*, 274–282. [CrossRef]

95. Fang, J.M.; Li, J.; Shi, J. An update on the diagnosis of gastroenteropancreatic neuroendocrine neoplasms. *World J. Gastroenterol.* **2022**, *28*, 1009–1023. [CrossRef] [PubMed]

96. Nanayakkara, J.; Tyryshkin, K.; Yang, X.; Wong, J.; Vanderbeck, K.; Ginter, P.S.; Scognamiglio, T.; Chen, Y.T.; Panarelli, N.; Cheung, N.K.; et al. Characterizing and classifying neuroendocrine neoplasms through microRNA sequencing and data mining. *NAR Cancer* **2020**, *2*, zcaa009. [CrossRef] [PubMed]

97. Geisler, L.; Mohr, R.; Lambrecht, J.; Knorr, J.; Jann, H.; Loosen, S.H.; Özdirik, B.; Luedde, T.; Hammerich, L.; Tacke, F.; et al. The Role of miRNA in the Pathophysiology of Neuroendocrine Tumors. *Int. J. Mol. Sci.* **2021**, *22*, 8569. [CrossRef]

98. Malczewska, A.; Kidd, M.; Matar, S.; Kos-Kudla, B.; Modlin, I.M. A Comprehensive Assessment of the Role of miRNAs as Biomarkers in Gastroenteropancreatic Neuroendocrine Tumors. *Neuroendocrinology* **2018**, *107*, 73–90. [CrossRef]

99. Li, A.; Yu, J.; Kim, H.; Wolfgang, C.L.; Canto, M.I.; Hruban, R.H.; Goggins, M. MicroRNA array analysis finds elevated serum miR-1290 accurately distinguishes patients with low-stage pancreatic cancer from healthy and disease controls. *Clin. Cancer Res.* **2013**, *19*, 3600–3610. [CrossRef]

100. Detassis, S.; Del Vescovo, V.; Grasso, M.; Masella, S.; Cantaloni, C.; Cima, L.; Cavazza, A.; Graziano, P.; Rossi, G.; Barbareschi, M.; et al. miR375-3p Distinguishes Low-Grade Neuroendocrine from Non-neuroendocrine Lung Tumors in FFPE Samples. *Front. Mol. Biosci.* **2020**, *7*, 86. [CrossRef]

101. Arvidsson, Y.; Rehammar, A.; Bergström, A.; Andersson, E.; Altiparmak, G.; Swärd, C.; Wängber, B.; Kristiansson, E.; Nilsson, O. miRNA profiling of small intestinal neuroendocrine tumors defines novel molecular subtypes and identifies miR-375 as a biomarker of patient survival. *Mod. Pathol.* **2018**, *31*, 1302–1317. [CrossRef]

102. Ruebel, K.; Leontovich, A.A.; Stilling, G.A.; Zhang, S.; Righi, A.; Jin, L.; Lloy, R.V. MicroRNA expression in ileal carcinoid tumors: Downregulation of microRNA-133a with tumor progression. *Mod. Pathol.* **2010**, *23*, 367–375. [CrossRef]

103. Korotaeva, A.; Mansorunov, D.; Apanovich, N.; Kuzevanova, A.; Karpukhin, A. MiRNA Expression in Neuroendocrine Neoplasms of Frequent Localizations. *Noncoding RNA* **2021**, *7*, 38. [CrossRef]

104. Knudsen, L.A.; Petersen, N.; Schwartz, T.W.; Egerod, K.L. The MicroRNA Repertoire in Enteroendocrine Cells: Identification of miR-375 as a Potential Regulator of the Enteroendocrine Lineage. *Endocrinology* **2015**, *156*, 3971–3983. [CrossRef] [PubMed]

105. Kwon, J.J.; Factora, T.D.; Dey, S.; Kota, J. A Systematic Review of miR-29 in Cancer. *Mol. Ther. Oncolytics* **2018**, *12*, 173–194. [CrossRef] [PubMed]

106. Özdirik, B.; Stueven, A.K.; Mohr, R.; Geisler, L.; Wree, A.; Knorr, J.; Demir, M.; Vucur, M.; Loosen, S.; Benz, F. Analysis of miR-29 Serum Levels in Patients with Neuroendocrine Tumors-Results from an Exploratory Study. *J. Clin. Med.* **2020**, *9*, 2881. [CrossRef] [PubMed]

107. Melone, V.; Salvati, A.; Palumbo, D.; Giurato, G.; Nassa, G.; Rizzo, F.; Palo, L.; Giordano, A.; Incoronato, M.; Vitale, M.; et al. Identification of functional pathways and molecular signatures in neuroendocrine neoplasms by multi-omics analysis. *J. Transl. Med.* **2022**, *20*, 306. [CrossRef] [PubMed]

108. Li, S.C.; Khan, M.; Caplin, M.; Meyer, T.; Öberg, K.; Giandomenico, V. Somatostatin Analogs Treated Small Intestinal Neuroendocrine Tumor Patients Circulating MicroRNAs. *PLoS ONE* **2015**, *10*, e0125553. [CrossRef]

109. Døssing, K.B.V.; Kjær, C.; Vikeså, J.; Binderup, T.; Knigge, U.; Culler, M.D.; Kjær, A.; Federspiel, B.; Friis-Hansen, L. Somatostatin Analogue Treatment Primarily Induce miRNA Expression Changes and Up-Regulates Growth Inhibitory miR-7 and miR-148a in Neuroendocrine Cells. *Genes* **2018**, *9*, 337. [CrossRef]

110. Zhang, H.; Lin, X.; Huang, Y.; Wang, M.; Cen, C.; Tang, S.; Dique, M.R.; Cai, L.; Luis, M.A.; Smollar, J.; et al. Detection Methods and Clinical Applications of Circulating Tumor Cells in Breast Cancer. *Front. Oncol.* **2021**, *11*, 652253. [CrossRef]

111. de Wit, S.; van Dalum, G.; Terstappen, L.W. Detection of circulating tumor cells. *Scientifica* **2014**, *2014*, 819362. [CrossRef]

112. Yang, C.; Xia, B.R.; Jin, W.L.; Lou, G. Circulating tumor cells in precision oncology: Clinical applications in liquid biopsy and 3D organoid model. *Cancer Cell Int.* **2019**, *19*, 341. [CrossRef] [PubMed]

113. Harouaka, R.; Kang, Z.; Zheng, S.Y.; Cao, L. Circulating tumor cells: Advances in isolation and analysis, and challenges for clinical applications. *Pharmacol. Ther.* **2014**, *141*, 209–221. [CrossRef] [PubMed]

114. Khan, M.S.; Tsigani, T.; Rashid, M.; Rabouhans, J.S.; Yu, D.; Luong, T.V.; Caplin, M.; Meyer, T. Circulating tumor cells and EpCAM expression in neuroendocrine tumors. *Clin. Cancer Res.* **2011**, *17*, 337–345. [CrossRef] [PubMed]

115. Khan, M.S.; Kirkwood, A.A.; Tsigani, T.; Garcia-Hernandez, J.; Hartley, J.A.; Caplin, M.E.; Meyer, T. Circulating tumor cells as prognostic markers in neuroendocrine tumors. *J. Clin. Oncol.* **2013**, *31*, 365–372. [CrossRef] [PubMed]

116. Khan, M.S.; Kirkwood, A.A.; Tsigani, T.; Lowe, H.; Goldstein, R.; Hartley, J.A.; Caplin, M.E.; Meyer, T. Early Changes in Circulating Tumor Cells Are Associated with Response and Survival Following Treatment of Metastatic Neuroendocrine Neoplasms. *Clin. Cancer Res.* **2016**, *22*, 79–85. [CrossRef]

117. Hsieh, J.C.; Chen, G.Y.; Jhou, D.D.; Chou, W.; Chun, N.; Hwang, T.; Ling, H.; Chu, H.; Wang, H.; Ten, T.; et al. The Prognostic Value of Circulating Tumor Cells in Asian Neuroendocrine Tumors. *Sci. Rep.* **2019**, *9*, 19917. [CrossRef]

118. Meyer, T.; Caplin, M.; Khan, M.S.; Toumpanakis, C.; Shetty, S.; Ramage, J.K.; Houchard, A.; Higgs, K.; Shah, T. Circulating tumour cells and tumour biomarkers in functional midgut neuroendocrine tumours. *J. Neuroendocrinol.* **2022**, *34*, e13096. [CrossRef]

119. Antonuzzo, L.; Meoni, G.; Di Costanzo, F. Are circulating tumor cells a new, valid prognostic marker in neuroendocrine tumors? *J. Clin. Oncol.* **2013**, *31*, 2518. [CrossRef]

120. Childs, A.; Steele, C.D.; Vesely, C.; Rizzo, F.M.; Ensell, L.; Lowe, H.; Dhami, P.; Vaikkinen, H.; Luong, T.V.; Conde, L.; et al. Whole-genome sequencing of single circulating tumor cells from neuroendocrine neoplasms. *Endocr. Relat. Cancer* **2021**, *28*, 631–644. [CrossRef]

121. Riihimäki, M.; Hemminki, A.; Sundquist, K.; Sundquist, J.; Hemminki, K. The epidemiology of metastases in neuroendocrine tumors. *Int. J. Cancer* **2016**, *139*, 2679–2686. [CrossRef]

122. Lin, D.; Shen, L.; Luo, M.; Zhang, K.; Li, J.; Yang, Q.; Zhu, F.; Zhou, D.; Zheng, S.; Chen, Y.; et al. Circulating tumor cells: Biology and clinical significance. *Signal Transduct. Target. Ther.* **2021**, *6*, 404. [CrossRef] [PubMed]

123. El Messaoudi, S.; Rolet, F.; Mouliere, F.; Thierry, A.R. Circulating cell free DNA: Preanalytical considerations. *Clin. Chim. Acta* **2013**, *424*, 222–230. [CrossRef] [PubMed]

124. Poulet, G.; Massias, J.; Taly, V. Liquid Biopsy: General Concepts. *Acta Cytologica* **2019**, *63*, 449–455. [CrossRef] [PubMed]

125. Mandel, P.; Metais, P. Les acides nucléiques du plasma sanguin chez 1 homme. *CR Seances Soc. Biol. Fil.* **1948**, *142*, 241–243.

126. Herrera-Martínez, A.D.; Hofland, L.J.; Gálvez Moreno, M.A.; Castaño, J.P.; de Herder, W.W.; Feelders, R.A. Neuroendocrine neoplasms: Current and potential diagnostic, predictive and prognostic markers. *Endocr. Relat. Cancer* **2019**, *26*, 157–179. [CrossRef]

127. Zakka, K.; Nagy, R.; Drusbosky, L.; Akce, M.; Wu, C.; Alese, O.B.; El-Rayes, B.F.; Kasi, P.M.; Mody, K.; Starr, J.; et al. Blood-based next-generation sequencing analysis of neuroendocrine neoplasms. *Oncotarget* **2020**, *11*, 1749–1757. [CrossRef]

128. Oxnard, G.R.; Thress, K.S.; Alden, R.S.; Lawrance, R.; Paweletz, C.P.; Cantarini, M.; Yang, J.C.H.; Barrett, J.C.; Jänne, P.A. Association Between Plasma Genotyping and Outcomes of Treatment with Osimertinib (AZD9291) in Advanced Non-Small-Cell Lung Cancer. *J. Clin. Oncol.* **2016**, *34*, 3375. [CrossRef]

129. Wang, Y.; Springer, S.; Zhang, M.; McMahon, K.W.; Kinde, I.; Dobbyn, L.; Ptak, J.; Brem, H.; Chaichana, K.; Gallia, G.L.; et al. Detection of tumor-derived DNA in cerebrospinal fluid of patients with primary tumors of the brain and spinal cord. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 9704–9709. [CrossRef]

130. Boons, G.; Vandamme, T.; Mariën, L.; Lybaert, W.; Roeyen, G.; Rondou, T.; Papadimitriou, K.; Janssens, K.; de Beeck, B.O.; Simoens, M.; et al. Longitudinal Copy-Number Alteration Analysis in Plasma Cell-Free DNA of Neuroendocrine Neoplasms is a Novel Specific Biomarker for Diagnosis, Prognosis, and Follow-up. *Clin. Cancer Res.* **2022**, *28*, 338–349. [CrossRef]

131. Gerard, L.; Garcia, J.; Gauthier, A.; Lopez, J.; Durand, A.; Hervieu, V.; Lemelin, A.; Chardon, L.; Landel, V.; Gibert, B.; et al. ctDNA in neuroendocrine carcinoma of gastroenteropancreatic origin or of unknown primary: The CIRCAN-NEC pilot study. *Neuroendocrinology* **2021**, *111*, 951–964. [CrossRef]

132. Oversoe, S.K.; Sorensen, B.S.; Tabaksblat, E.M.; Gronbaek, H.; Kelsen, J. Cell-Free DNA and Clinical Characteristics in Patients with Small Intestinal or Pancreatic Neuroendocrine Tumors. *Neuroendocrinology* **2022**, *112*, 43–50. [CrossRef] [PubMed]

133. Boons, G.; Vandamme, T.; Peeters, M.; Beyens, M.; Driessen, A.; Janssens, K.; Zwaenepoel, K.; Roeyen, G.; Van Camp, G.; Op de Beeck, K. Cell-Free DNA From Metastatic Pancreatic Neuroendocrine Tumor Patients Contains Tumor-Specific Mutations and Copy Number Variations. *Front. Oncol.* **2018**, *8*, 467. [CrossRef]

134. Sozzi, G.; Conte, D.; Leon, M.; Cirincione, R.; Roz, L.; Ratcliffe, C.; Roz, E.; Cirenei, N.; Bellomi, M.; Pelosi, G.; et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. *J. Clin. Oncol.* **2003**, *21*, 3902–3908. [CrossRef] [PubMed]

135. Kim, K.; Shin, D.G.; Park, M.K.; Baik, S.H.; Kim, T.H.; Kim, S.; Lee, S. Circulating cell-free DNA as a promising biomarker in patients with gastric cancer: Diagnostic validity and significant reduction of cfDNA after surgical resection. *Ann. Surg. Treat. Res.* **2014**, *86*, 136–142. [CrossRef] [PubMed]

136. Garcia, J.; Forestier, J.; Dusserre, E.; Wozny, A.S.; Geiguer, F.; Merle, P.; Tissot, C.; Ferraro-Peyret, C.; Jones, F.S.; Edelstein, D.L.; et al. Cross-platform comparison for the detection of RAS mutations in cfDNA (ddPCR Biorad detection assay, BEAMing assay, and NGS strategy). *Oncotarget* **2018**, *9*, 21122–21131. [CrossRef] [PubMed]

137. Hijioka, S.; Hosoda, W.; Matsuo, K.; Ueno, M.; Furukawa, M.; Yoshitomi, H.; Kobayashi, N.; Ikeda, M.; Ito, T.; Nakamori, S.; et al. Rb Loss and KRAS Mutation Are Predictors of the Response to Platinum-Based Chemotherapy in Pancreatic Neuroendocrine Neoplasm with Grade 3: A Japanese Multicenter Pancreatic NEN-G3 Study. *Clin. Cancer Res.* **2017**, *23*, 4625–4632. [CrossRef]

138. Luo, H.; Wei, W.; Ye, Z.; Zheng, J.; Xu, R.H. Liquid Biopsy of Methylation Biomarkers in Cell-Free DNA. *Trends. Mol. Med.* **2021**, *27*, 482–500. [CrossRef]

139. Choi, I.S.; Estecio, M.R.; Nagano, Y.; Kim, D.H.; White, J.A.; Yao, J.C.; Issa, J.P.; Rashid, A. Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod. Pathol.* **2007**, *20*, 802–810. [CrossRef]

140. Stefanoli, M.; La Rosa, S.; Sahnane, N.; Romualdi, C.; Pastorino, R.; Marando, A.; Capella, C.; Sessa, F.; Furlan, D. Prognostic relevance of aberrant DNA methylation in g1 and g2 pancreatic neuroendocrine tumors. *Neuroendocrinology* **2014**, *100*, 26–34. [CrossRef]

141. Stricker, I.; Tzivras, D.; Nambiar, S.; Wulf, J.; Liffers, S.T.; Vogt, M.; Verdoodt, B.; Tannapfel, A.; Mirmohammadsadegh, A. Site- and grade-specific diversity of LINE1 methylation pattern in gastroenteropancreatic neuroendocrine tumours. *Anticancer Res.* **2012**, *32*, 3699–3706.

142. Colao, A.; de Nigris, F.; Modica, R.; Napoli, C. Clinical Epigenetics of Neuroendocrine Tumors: The Road Ahead. *Front. Endocrinol.* **2020**, *11*, 604341. [CrossRef]

143. Mettler, E.; Fottner, C.; Bakhshandeh, N.; Trenkler, A.; Kuchen, R.; Weber, M.M. Quantitative Analysis of Plasma Cell-Free DNA and Its DNA Integrity and Hypomethylation Status as Biomarkers for Tumor Burden and Disease Progression in Patients with Metastatic Neuroendocrine Neoplasias. *Cancers* **2022**, *14*, 1025. [CrossRef] [PubMed]

144. Cote, G.J.; Evers, C.; Hu, M.I.; Grubbs, E.G.; Williams, M.D.; Hai, T.; Duose, D.Y.; Houston, M.R.; Bui, J.H.; Mehrotra, M.; et al. Prognostic Significance of Circulating RET M918T Mutated Tumor DNA in Patients with Advanced Medullary Thyroid Carcinoma. *J. Clin. Endocrinol. Metab.* **2017**, *102*, 3591–3599. [CrossRef]

145. Rizzo, F.M.; Meyer, T. Liquid Biopsies for Neuroendocrine Tumors: Circulating Tumor Cells, DNA, and MicroRNAs. *Endocrinol. Metab. Clin. N. Am.* **2018**, *47*, 471–483. [CrossRef]

146. Moschovis, D.; Gazouli, M.; Tzouvala, M.; Vezakis, A.; Karamanolis, G. Long non-coding RNA in pancreatic adenocarcinoma and pancreatic neuroendocrine tumors. *Ann. Gastroenterol.* **2017**, *30*, 622–628. [CrossRef] [PubMed]

147. Xu, S.S.; Xu, H.X.; Wang, W.Q.; Li, S.; Li, H.; Li, T.J.; Zhang, W.H.; Liu, L.; Yu, X.J. Tumor-infiltrating platelets predict postoperative recurrence and survival in resectable pancreatic neuroendocrine tumor. *World J. Gastroenterol.* **2019**, *25*, 6248–6257. [CrossRef] [PubMed]

148. Hinterleitner, M.; Sipos, B.; Wagner, V.; Grottenthaler, J.M.; Lauer, U.M.; Zender, L.; Hinterleitner, C. Platelet-Expressed Synaptophysin (pSyn) as Novel Biomarker in Neuroendocrine Malignancies. *Cancers* **2021**, *13*, 2286. [CrossRef]

149. La Salvia, A.; Soldevilla, B.; Lens-Pardo, A.; Lopez-Lopez, A.; Carretero, C.; Capdevila, J.; Vinuales, B.M.; Jimenez-Fonseca, P.; Lopez-Gonzalvez, A.; Espinosa-Olarte, P.; et al. Metabolomic profile of extra-pancreatic NET: A novel prognostic tool? In Proceedings of the 19th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, Barcelona, Spain, 10–11 March 2022.

150. Soldevilla, B.; López-López, A.; Lens-Pardo, A.; Carretero-Puche, C.; Lopez-Gonzalvez, A.; La Salvia, A.; Gil-Calderon, B.; Riesco-Martinez, M.C.; Espinosa-Olarte, P.; Sarmentero, J.; et al. Comprehensive Plasma Metabolomic Profile of Patients with Advanced Neuroendocrine Tumors (NETs). Diagnostic and Biological Relevance. *Cancers* **2021**, *13*, 2634. [CrossRef]

151. Marien, L.; Boons, G.; Vandamme, T.; Lybaert, W.; Roeyen, G.; Papadimitriou, K.; Janssens, K.; Op de Beeck, B.; Demey, W.; van Camp, G.; et al. Longitudinal copy number alteration analysis in plasma cell-free DNA of neuroendocrine neoplasms is a novel specific biomarker for diagnosis, prognosis and follow-up. In Proceedings of the 19th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, Barcelona, Spain, 10–11 March 2022.

152. Garcia Alvarez, A.; Hernando, J.; Jimenez-Valero, G.; Martinez, A.; Fazio, N.; Lopez, C.; Teule, A.; Valle, J.W.; Tafuto, S.; Custodio, A.; et al. Identification of proangiogenic plasmatic biomarkers in patients with advanced grade 1/2 pancreatic (pan) and gastrointestinal (gi) neuroendocrine tumors (NETs) treated with Lenvatinib: A subanalysis from the TALENT phase II clinical trial. In Proceedings of the 19th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, Barcelona, Spain, 10–11 March 2022.

153. Gohil, S.; Hastings, R.; Page, K.; Iwuji, C.; Richards, C.; Shaw, J.; Levy, M. Circulating cell free tumor DNA as a biomarker in patients with neuroendocrine neoplasms. In Proceedings of the 19th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, Barcelona, Spain, 10–11 March 2022.

154. Levy, S.; Kilgallen, A.B.; Korse, C.M.; Oerlemans, M.J.F.; Sluijter, J.P.G.; Valk, G.D.; van Laake, L.W.; Tesselaar, M.E.T. Serum Activin A is not associated with carcinoid heart disease (CHD) in patients with neuroendocrine tumors (NET). In Proceedings of the 19th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, Barcelona, Spain, 10–11 March 2022.

155. Schalin-Jantti, C.; Kjellman, M.; Knigge, U.; Gronbaek, H.; Thiis-Evensen, E.; Welin, S.; Sorbye, H.; Schneider, M.; Belusa, R.; The Nordic NET Biomarker Group. Factors at time of diagnosis associated with progressive or stable disease in patients with Small Intestinal Neuroendocrine Tumors (SI-NETs). In Proceedings of the 19th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, Barcelona, Spain, 10–11 March 2022.

156. Van Treijen, M.; Korse, C.M.; Verbeek, W.; Tesselaar, M.E.T.; Valk, G.D. Serial liquid biopsies—The NETest—in gastroenteropancreatic NET surveillance. In Proceedings of the 19th Annual ENETS Conference for the Diagnosis and Treatment of Neuroendocrine Tumor Disease, Barcelona, Spain, 10–11 March 2022.

157. Öberg, K. Molecular Genomic Blood Biomarkers for Neuroendocrine Tumors: The Long and Winding Road from Berzelius and Bence Jones to a Neuroendocrine Destination. *Neuroendocrinology* **2021**, *111*, 297–303. [CrossRef]

158. Kidd, M.; Kitz, A.; Drozdov, I.; Modlin, I. Neuroendocrine Tumor Omic Gene Cluster Analysis Amplifies the Prognostic Accuracy of the NETest. *Neuroendocrinology* **2021**, *111*, 490–504. [CrossRef]

159. Rindi, G.; Wiedenmann, B. Neuroendocrine neoplasia goes molecular—Time for a change. *Nat. Rev. Clin. Oncol.* **2019**, *16*, 149–150. [CrossRef] [PubMed]

160. Starzyńska, T.; Londzin-Olesik, M.; Bednarczuk, T.; Bolanowski, M.; Borowska, M.; Chmielik, E.; Ćwikła, J.B.; Foltyn, W.; Gisterek, I.; Handkiewicz-Junak, D.; et al. Colorectal neuroendocrine neoplasms—Update of the diagnostic and therapeutic guidelines (recommended by the Polish Network of Neuroendocrine Tumours) [Nowotwory neuroendokrynne jelita grubego—Uaktualnione zasady diagnostyki i leczenia (rekomendowane przez Polską Sieć Guzów Neuroendokrynnych)]. *Endokrynol. Pol.* **2022**, *73*, 584–611. [CrossRef] [PubMed]

161. Bednarczuk, T.; Zemczak, A.; Bolanowski, M.; Borowska, M.; Chmielik, E.; Ćwikła, J.B.; Foltyn, W.; Gisterek, I.; Handkiewicz-Junak, D.; Hubalewska-Dydejczyk, A.; et al. Neuroendocrine neoplasms of the small intestine and the appendix—Update of the diagnostic and therapeutic guidelines (recommended by the Polish Network of Neuroendocrine Tumours) [Nowotwory neuroendokrynne jelita cienkiego i wyrostka robaczkowego—Uaktualnione zasady diagnostyki i leczenia (rekomendowane przez Polską Sieć Guzów Neuroendokrynnych)]. *Endokrynol. Pol.* **2022**, *73*, 549–583. [CrossRef]

162. Kos-Kudła, B.; Rosiek, V.; Borowska, M.; Bednarczuk, T.; Bolanowski, M.; Chmielik, E.; Ćwikła, J.B.; Foltyn, W.; Gisterek, I.; Handkiewicz-Junak, D.; et al. Pancreatic neuroendocrine neoplasms—Update of the diagnostic and therapeutic guidelines (recommended by the Polish Network of Neuroendocrine Tumours) [Nowotwory neuroendokrynne trzustki—Uaktualnione zasady diagnostyki i leczenia (rekomendowane przez Polską Sieć Guzów Neuroendokrynnych)]. *Endokrynol. Pol.* **2022**, *73*, 491–548. [CrossRef]

163. Grozinsky-Glasberg, S.; Davar, J.; Hofland, J.; Dobson, R.; Prasad, V.; Pascher, A.; Denecke, T.; Tesselaar, M.; Panzuto, F.; Albåge, A.; et al. European Neuroendocrine Tumor Society (ENETS) 2022 Guidance Paper for Carcinoid Syndrome and Carcinoid Heart Disease. *J. Neuroendocrinol.* **2022**, *34*, e13146. [CrossRef] [PubMed]

Article

The Clinical Characteristics of Pheochromocytomas and Paragangliomas with Negative Catecholamines

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Abstract: Pheochromocytomas and paragangliomas (PPGLs) associated with negative catecholamines are not uncommon. However, few studies have examined clinical features of patients with these tumors. In the absence of available data, it is difficult to identify characteristics of patients with potential PPGLs and normal serum and urine screens. Therefore, an analysis of patients with PPGLs was conducted retrospectively to compare the clinical features of patients with positive and negative catecholamines. This study included 214 patients, including 69 patients with negative catecholamines. Prevalence rates of diabetes ($p < 0.001$) and hypertension ($p < 0.001$) were lower and tumor diameter ($p < 0.001$) was smaller in the negative-catecholamine group compared with the positive-catecholamine group. Multivariable logistic regression analysis showed that extra-adrenal PPGLs were independently positively associated with negative catecholamines ($p = 0.004$); hypertension ($p = 0.001$) and tumor diameter ($p = 0.016$) were independently negatively associated with negative catecholamines. There was no significant difference in tumor recurrence between the two groups (mean follow-up, 20.54 ± 11.83 months) ($p = 0.44$). The results demonstrated that PPGL patients with negative catecholamines were more likely to have extra-adrenal tumors and less likely to have comorbidities, and these patients should also be closely monitored for tumor recurrence.

Keywords: pheochromocytomas; paragangliomas; catecholamine

1. Introduction

Pheochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumors. Pheochromocytomas (PHEOs) originate from chromaffin cells of the adrenal cortex, and paragangliomas (PGLs) originate from extra-adrenal chromaffin cells of the sympathetic paravertebral ganglia located in the thorax, abdomen, pelvis, and from parasympathetic ganglia located along the glossopharyngeal and vagal nerves in the neck and at the base of the skull [1]. The combined incidence is approximately 0.57 cases per 100,000 person-years [2]. Symptoms such as headache, palpitations, and sweating are caused by catecholamines produced by these tumors [3]. PPGLs should be diagnosed and treated as soon as possible because incorrectly treated PPGLs can cause life-threatening complications [4].

As PPGLs secrete catecholamines, the diagnostic biochemical tests for these tumors involve the detection of these hormones. According to current clinical practice guidelines, measurements of plasma or urinary catecholamines should be performed during biochemical screening for PPGLs, and there is no recommendation regarding which test should be preferred [1]. Even though these tests are with high sensitivity, for example, the sensitivity of plasma free metanephrines to diagnose PPGLs has been reported as between 96 and 99% [5], there are indeed many patients with PPGLs who do not exhibit elevated catecholamines. It may pose a problem for clinicians who mistakenly believe they have ruled out PPGLs. However, there is little information in the current literature concerning the clinical features of catecholamine-negative PPGLs, with the majority being case studies of single patients [6–8]. Therefore, the objective of this study was to compare clinical characteristics of PPGL patients with positive and negative catecholamine levels to provide more information for clinicians to better understand this clinical population.

2. Materials and Methods

All consecutive adult patients with PPGLs who underwent surgical resection and had their diagnosis confirmed by pathological examinations from January 2018 to June 2020 in the Peking Union Medical College Hospital were retrospectively enrolled. The electronic medical files of patients were reviewed. Clinical history data, preoperative biochemical examination results, and tumor diameters and locations were obtained from the electronic medical record. A total of 313 patients were eligible for study inclusion; we then excluded 58 patients with no catecholamine information, 25 patients who presented to our hospital due to recurrence or metastasis of PPGLs after treatment in other hospitals, and 16 patients with incomplete clinical data. In total, 214 were included for analysis.

Patients were grouped according to catecholamine concentration measurements. Negative catecholamine was defined as when 24 h urinary catecholamine (epinephrine and norepinephrine), plasma metanephrine, and plasma normetanephrine concentrations did not exceed their respective reference limits. Positive catecholamine was defined as an abnormal elevation of the 24 h urinary catecholamine (epinephrine and norepinephrine), plasma metanephrine, or plasma normetanephrine. The diagnosis of hypertension and diabetes was made on based on patient history and preoperative blood pressure and blood glucose measurements, respectively. The patterns of hypertension in patients with PPGLs comprised sustained, paroxysmal, and mixed patterns [3]. Recurrence was defined as local relapse detected on computed tomography, magnetic resonance imaging, or functional imaging. Metastatic PPGL was defined as the recurrence at sites without chromaffin tissue [9]. All recorded laboratory indicators were the results of the patients before surgery. Measurements of plasma normetanephrine, plasma metanephrine, and 24 h urinary catecholamines were by mass spectrometry. Plasma metanephrine and plasma normetanephrine were measured after the patients maintained a supine position for at least 30 min [1]. Factors that affect catecholamine levels, such as caffeine, tricyclic antidepressants, phenoxybenzamine, sympathomimetics, and monoamine oxidase inhibitors, were discontinued at least 24 h before blood samples were obtained [1,10,11]. The tumor diameters were determined on the basis of the pathological specimens. Most PHEOs were resected with minimally invasive adrenalectomy; however, open resection was performed for large tumors (>6 cm). Most PGLs were resected with open surgery; however, laparoscopic resection was performed for small tumors in surgically favorable locations [1]. All patients with hormonally functional PPGLs underwent preoperative blockade, and the α-adrenergic receptor blockers were the first choice. The β-adrenergic receptor blockers were indicated only after administration of α-adrenergic receptor blockers [1]. If patients with negative catecholamines had positive functional imaging findings, they also underwent preoperative preparation as described above. If these patients were with negative functional imaging results, a decision about whether to use preoperative preparation was made by multidisciplinary teamwork [12].

The study was approved by the ethics committee of Peking Union Medical College Hospital and was conducted in accordance with the Declaration of Helsinki. The in-formed consent requirement was waived because all data were anonymized.

Statistical methods

Histograms and normal quantile–quantile plots were used to assess normality. Continuous data were reported as the mean $\pm$ standard or median (25th, 75th percentiles), and they were compared between the groups by Student's *t*-test or the rank-sum test. Categorical variables are presented as numbers (percentages) and were compared using the Pearson's chi-square test or Fisher's exact test as appropriate. Parameters with $p < 0.1$ in the univariate logistic regression analysis were included in the multivariate logistic regression analysis. Two-sided *p* value < 0.05 was considered as statistically significant. Statistical analyses were performed using SPSS statistical software, version 25.0 (IBM Corp., Armonk, NY, USA). GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA) was used to perform receiver operating characteristic (ROC) curve analysis.

3. Results

Among the 214 study patients, 69 patients had negative catecholamine levels. The patients' clinical characteristics are summarized in Table 1. The mean age of the entire study population was 46.01 $\pm$ 12.95 years. Hypertension and diabetes accounted for 63.6% and 26.6% of the patients, respectively. Incidentaloma occurred in 79 patients (36.9%) in the entire cohort. Tumor location was extra-adrenal in 93 patients (43.5%) and adrenal in 121 (56.5%). Among extra-adrenal tumors, in 25 patients (26.9%) they were located in head and neck, and in 68 (73.1%) they were located in the thorax or abdomen. Open resection and minimally invasive adrenalectomy were performed in 142 patients (66.4%) and 72 patients (33.6%), respectively.

Table 1. Clinical characteristics of patients with pheochromocytoma and paragangliomas.

Variable	All ($n = 214$)
Age, years ($n = 214$)	46.01 $\pm$ 12.95
Female, % ($n = 214$)	112(52.3)
Diabetes, % ($n = 214$)	57(26.6)
Hypertension, % ($n = 214$)	136(63.6)
BMI, kg/m^2 ($n = 214$)	24.37 $\pm$ 3.27
Metabolic parameters	
Glucose, mmol/L ($n = 214$)	5.4(4.7, 6.53)
Total cholesterol, mmol/L ($n = 211$)	4.49(4.02, 5.23)
Triglyceride, mmol/L ($n = 211$)	1.25(0.85, 1.79)
LDL-c, mmol/L ($n = 210$)	2.76 $\pm$ 0.76
Plasma metanephrine, nmol/L ($n = 118$)	0.18(0.1, 2.59)
Plasma normetanephrine, nmol/L ($n = 118$)	2.50(0.79, 5.98)
24hU-E, μg/24 h ($n = 214$)	4.22(2.81, 17.51)
24hU-NE, μg/24 h ($n = 214$)	56.88(31.81,188.01)
24hU-DA, μg/24 h ($n = 214$)	232.15(186.74, 296.01)
Tumor characteristics ($n = 214$)	
Adrenal PPGL, %	121(56.5)
Extra-adrenal PPGL, %	93(43.5)
Tumor diameter (cm)	5.0(4.0, 6.53)

PPGL, pheochromocytoma and paraganglioma; BMI, body mass index; 24hU-E: 24 h urine epinephrine; 24hU-NE: 24 h urine norepinephrine; 24hU-DA: 24 h urine dopamine; LDL-c, low-density lipoprotein cholesterol. Reference range: plasma metanephrine: <0.5nmol/L; plasma normetanephrine: <0.9 nmol/L; 24hU-E: 1.74–6.42 μg/24 h; 24hU-NE: 16.69–40.65 μg/24 h; 24hU-DA: 120.93–330.59 μg/24 h.

The clinical characteristics of patients with negative and positive catecholamines are summarized in Table 2. Age, sex, and BMI were not significantly different between the two groups. Fewer patients in the negative-catecholamine group had hypertension and diabetes compared with the positive-catecholamine group (43.5% vs. 73.1%, $p < 0.001$ and 10.1% vs. 34.5%, $p < 0.001$, respectively). Concentrations of total cholesterol, triglycerides,

and low-density lipoprotein cholesterol did not significantly differ between the groups. In the negative-catecholamine group, median concentrations of plasma metanephrine, plasma normetanephrine, 24 h urine epinephrine, and 24 h urine norepinephrine were 0.1 nmol/L, 0.27 nmol/L, 3.37 µg/24 h, and 28.39 µg/24 h, respectively. Corresponding concentrations in the positive-catecholamine group were 0.39 nmol/L, 3.77 nmol/L, 5.68 µg/24 h, and 126.00 µg/24 h, respectively. Extra-adrenal PPGLs were more frequent in the negative-catecholamine group compared with the positive-catecholamine group (65.2% vs. 33.1%, $p < 0.001$). In patients with head and neck PPGLs, 2 patients (4.2%) were in the positive-catecholamine group and 23 patients (51.1%) were in the negative-catecholamine group ($p < 0.001$). In patients with thoracic or abdominal PPGLs, 46 patients (95.8%) were in the positive-catecholamine group and 22 patients (48.9%) were in the negative-catecholamine group ($p < 0.001$). Tumor diameter in the negative-catecholamine group was significantly smaller than that in the positive-catecholamine group (4.0 (3.0, 6.0) vs. 5.5 (4.5, 7.0) cm, $p < 0.001$).

Table 2. Clinical characteristics of the patients in the positive- and negative-catecholamine groups.

Variable	Negative-Catecholamine Group ($n = 69$)	Positive-Catecholamine Group ($n = 145$)	*p* Value
Age, years ($n = 214$)	47.91 ± 12.53	45.10 ± 13.09	0.138
Female, % ($n = 214$)	41(59.4)	71(49.0)	0.152
Diabetes, % ($n = 214$)	7(10.1)	50(34.5)	<0.001
Hypertension, % ($n = 214$)	30(43.5)	106(73.1)	<0.001
BMI, kg/m^2 ($n = 214$)	24.63 ± 2.98	24.26 ± 3.40	0.439
Metabolic parameters			
Glucose, mmol/L ($n = 214$)	4.9(4.6, 5.65)	5.6(4.9, 6.75)	0.001
Total cholesterol, mmol/L ($n = 211$)	4.38(3.96, 4.92)	4.55(4.03, 5.34)	0.103
Triglyceride, mmol/L ($n = 211$)	1.36(0.90, 1.86)	1.23(0.76, 1.76)	0.36
LDL-c, mmol/L ($n = 210$)	2.63 ± 0.74	2.81 ± 0.77	0.106
Tumor characteristics ($n = 214$)			
Adrenal PPGL, %	24(34.8)	97(66.9)	<0.001
Extra-adrenal PPGL, %	45(65.2)	48(33.1)	<0.001
Tumor diameter (cm)	4.0(3.0, 6.0)	5.5(4.5, 7.0)	<0.001

PPGL, pheochromocytoma and paraganglioma; BMI, body mass index; LDL-c, low-density lipoprotein cholesterol.

In the univariate logistic regression analysis, extra-adrenal PPGLs were positively associated with negative catecholamines (odds ratio (OR): 3.789, 95% confidence interval (95% CI): 2.071–6.933; $p < 0.001$). Diabetes, hypertension, and tumor diameter were negatively associated with negative catecholamines (OR: 0.215, 95% CI: 0.091–0.504, $p < 0.001$; OR: 0.283, 95% CI: 0.155–0.516, $p < 0.001$; and OR: 0.77, 95% CI: 0.662–0.895, $p = 0.001$, respectively). These results are summarized in Table 3. According to the results of the univariate logistic regression analysis, diabetes, hypertension, total cholesterol, extra-adrenal PPGL, and tumor diameter were included in the multivariate logistic regression analysis. The results showed that extra-adrenal PPGL (OR, 2.724; 95% CI: 1.382–5.372; $p = 0.004$) was independently positively associated with negative catecholamines; hypertension (OR, 0.305, 95% CI: 0.155–0.600, $p = 0.001$) and tumor diameter (OR, 0.826, 95% CI: 0.707–0.966, $p = 0.016$) were independently negatively associated with negative catecholamines. We used ROC curves to determine the diagnostic potential of tumor diameter for PPGLs with negative catecholamines. The area under the curve was 0.660 (95% CI: 0.577–0.743; $p < 0.001$), and the cutoff value was 4.85 cm (Figure 1).

Table 3. Results of the univariate logistic regression.

Variable	p	OR	95% CI
Age	0.139	1.017	0.994–1.040
Female	0.153	1.526	0.854–2.727
Diabetes	<0.001	0.215	0.091–0.504
Hypertension	<0.001	0.283	0.155–0.516
BMI	0.437	1.035	0.948–1.130
Total cholesterol	0.09	0.759	0.551–1.044
Triglyceride	0.747	0.963	0.768–1.209
LDL-c	0.108	0.725	0.489–1.073
Extra-adrenal PPGL	<0.001	3.789	2.071–6.933
Tumor diameter	0.001	0.77	0.662–0.895

PPGL, pheochromocytoma and paraganglioma; BMI, body mass index; LDL-c, low-density lipoprotein cholesterol; OR, odds ratio; 95% CI, 95% confidence interval.

AUC = 0.660; $p < 0.001$
95%CI: 0.577–0.743
Cut-off value = 4.85 cm
Sensitivity = 61%
Specificity = 72%
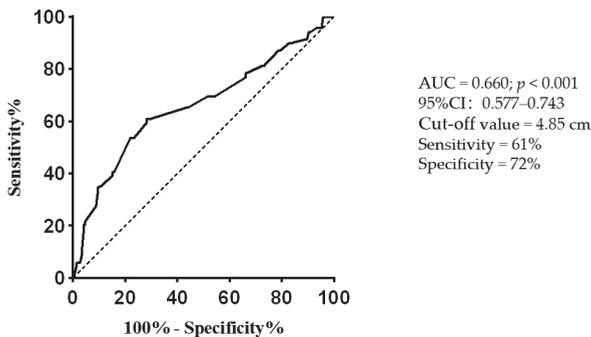
Figure 1. Receiver operating characteristic curve evaluating the diagnostic potential of tumor diameter for predicting PPGLs with negative catecholamines. AUC: area under the curve; PPGLs: pheochromocytomas and paragangliomas.

In this study, 180 patients were followed up for a mean of 20.54 ± 11.83 months, including 61 in the negative-catecholamine group and 119 in the positive-catecholamine group. Among them, five patients developed disease recurrence, namely three in the negative-catecholamine group and two in the positive-catecholamine group. There was no significant difference in tumor recurrence rates between the groups ($p = 0.44$). Three patients were diagnosed with metastases during the follow-up, and all were in the positive-catecholamine group. One patient died because of hypertension in the positive-catecholamine group.

4. Discussion

In our study, a positive association was found between extra-adrenal PPGLs and negative catecholamines, and there was no significant difference in early tumor recurrence rates between the two groups. Additionally, comorbidities were less frequent and tumor diameter was smaller in the negative-catecholamine group. This study provided useful information for clinicians to understand the PPGL patients with negative catecholamines, which was very helpful for diagnosis and follow-up of patients with PPGLs.

A previous study of 42 patients presenting with adrenal incidentaloma revealed 14 cases of PHEO, with 3 (21%) of these exhibiting borderline urine or serum metanephrine concentrations [13]. Another study from Italy revealed that 14% of the patients with PHEOs had negative urine catecholamines [14]. In Kawashima et al.'s cohort [15], the prevalence of patients with PPGLs and negative urine catecholamine results was 6.2%. In Heavner et al.'s study [6], 9% of patients with PHEOs had negative markers preoperatively. On the basis of these findings, the exact proportion of negative catecholamines in patients with PPGLs is not yet clear. Two factors may explain the wide range of reported rates: study population and definition of negative catecholamine concentrations. The evaluated population in our

study comprised patients with PPGLs; however, some previous studies evaluated patients with PHEOs only [6,14]. Furthermore, the definition of negative catecholamines varied in previous studies in accordance with testing conditions at the different medical facilities where the studies were conducted. In our study, negative catecholamine was defined as plasma metanephrine, plasma norepinephrine, and urinary catecholamine concentrations not exceeding their respective upper reference limits. In Kawashima et al.'s study [15], negative catecholamine was defined as when the levels of urinary metanephrine and normetanephrine did not exceed their upper reference limits. Large-scale, well-defined, and well-targeted studies are needed to address this issue; however, because of the rarity of PPGLs, performing these studies will be a great challenge.

In the present study, extra-adrenal PPGLs were significantly associated with negative catecholamines, and this result was similar with Kawashima et al.'s study [15]. An association between negative catecholamines and extra-adrenal PPGLs is implied by the high proportion of extra-adrenal PPGLs in patients in the negative-catecholamine group. In PPGLs with the *SDHB* mutation, tyrosine hydroxylase is sometimes absent, resulting in PPGL with biochemical silence [16]. Moreover, biochemically silent PPGLs have been associated with *SDHD* mutations in a previous study [17]. A recent paper describing the natural history and management of familial PGL syndrome type 1 also reported that negative biochemical results occurred in the patients with *SDHD* mutations [18]. In addition, according to Neumann et al.'s study, patients with *SDHB/SDHD* mutations were significantly more likely to develop extra-adrenal PPGL than those without [19]. According to the results of above studies, the tumor locations and catecholamine secretion may be associated with the type of gene mutation.

In a recent systematic review reporting patients with PPGLs treated with Sunitinib, almost half of the patients with malignant PPGLs did not have excess catecholamine secretion, while the remaining patients were with elevated catecholamines [20]. This phenomenon suggests that it is very interesting to explore the relationship between catecholamine secretion and metastatic progression of PPGLs. In Kawashima et al.'s study [15], PPGLs with negative catecholamines were associated with metastatic disease. In contrast to Kawashima et al.'s results [15], Heavner et al. [6] reported there were no metastatic PPGLs in patients with negative catecholamines, whereas there were nine metastatic cases in patients with positive catecholamines. Another study also reported that catecholamine concentrations were higher in patients with metastatic PPGLs than non-metastatic PPGLs [21]. In our study, after the short-term follow-up, only three patients were diagnosed with metastatic PPGLs, and all were in the catecholamine-positive group. Compared with previous reports [15,22,23], the proportion of metastatic PPGLs was lower in this study. The possible reasons for this difference are as follows: First, we excluded patients who presented with recurrence or metastasis of PPGL after treatment in other hospitals before analysis. Second, as metastatic PPGLs often become evident several years after initial diagnosis, the lower metastatic prevalence in this study may be due in part to the short-term follow-up. Nonetheless, the proportion of tumor recurrence between the two groups was not significantly different, suggesting that it is essential to closely monitor patients in the catecholamine-negative group for tumor recurrence, just as patients with positive catecholamines.

Several previous studies have reported a positive correlation between tumor size and catecholamine concentrations [24–26]. In this study, we also found that tumor diameter in the patients in the negative-catecholamine group was smaller than that in the positive-catecholamine group. Although tumor diameter was smaller in patients with negative catecholamines, existing literature has indicated that caution should be exercised regarding complications when resecting these tumors. In one case report, a hypertensive episode occurred during resection of an incidentally discovered adrenal lesion in a patient without elevated metanephrine concentration, and PHEO was later diagnosed [7]. Despite successful treatment, this case illustrates that complications may still occur during surgical resection of tumors with negative catecholamines.

In our study, hypertension and diabetes were less frequent in the negative catecholamine group than the positive catecholamine group, which was expected owing to the effect of catecholamines on blood pressure and glucose metabolism [27–29]. Catecholamines also affect body weight during hypermetabolic and proinflammatory states [30]. As a result of a comparison between patients with negative catecholamines and patients with catecholamine-positive PPGLs, Heavner et al. [6] reported that BMI was higher in patients with negative catecholamines; however, Kawashima et al. [15] did not find a difference in BMI between their negative- and positive-catecholamine groups, and the results in this study were consistent with Kawashima et al.'s. The difference between the BMI in the above studies may be due to the different prevalence of obesity between Asians and Americans.

Anatomical documentation of the tumor is necessary to diagnose PPGLs, and hormonal tests for catecholamines are helpful in the diagnosis of them [11]. Current Endocrine Society Guidelines [1] suggests annual biochemical surveillance for PPGL patients. According to Puliani et al.'s suggestions [18], in PPGL patients with negative biochemical results and *SDHD* mutations, periodic follow-up should include an annual biochemical and ultrasonographic screening and biannual neck-mediastinum magnetic resonance examination. Based on our experience, for catecholamine-negative patients, we also recommend annual biochemical testing and ultrasonographic screening, as well as biannual magnetic resonance imaging to assess recurrence and metastasis.

This study has several limitations. First, bias was inevitable because of the retrospective and single-center study design. Second, plasma metanephrine and plasma normetanephrine concentrations were not measured in all patients. However, not all hospitals have the ability to measure plasma-free catecholamines, while measurement of urine catecholamines is common and feasible. Third, owing to the lack of genetic screening, we could not confirm a relationship between genotype and catecholamines. Fourth, there was no reliable method for dopamine-producing tumors. The plasma methoxytyramine measurement was not available in our medical institution; although urinary dopamine was collected, the majority of it is synthesized in the renal tubules from circulating Dopa. Therefore, urinary dopamine is not a reliable indicator of dopamine-producing tumors. As tumors that produce dopamine predominantly or exclusively are rare [31–33], the results in our study can still be used for the assessments of most PPGLs.

5. Conclusions

The existence of catecholamine-negative PPGLs has been established, and they are not uncommon. Negative first-line catecholamine testing does not necessarily rule out a diagnosis of PPGLs. PPGL patients with negative catecholamines had an increased likelihood of having extra-adrenal lesions and a lower likelihood of having comorbidities. In addition, patients with preoperative negative catecholamines should be closely monitored for tumor recurrence.

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Institutional Review Board Statement: This study was approved by the ethics committee of Peking Union Medical College Hospital (approval number: JS-2898 (accessed on 27 April 2021)).

Informed Consent Statement: Owing to the retrospective nature of the study and that all data were anonymized and de-identified, the requirement for informed consent in this study was waived.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lenders, J.W.; Duh, Q.Y.; Eisenhofer, G.; Gimenez-Roqueplo, A.P.; Grebe, S.K.; Murad, M.H.; Naruse, M.; Pacak, K.; Young, W.F., Jr. Pheochromocytoma and paraganglioma: An endocrine society clinical practice guideline. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 1915–1942. [CrossRef]
2. Berends, A.M.A.; Buitenwerf, E.; de Krijger, R.R.; Veeger, N.; van der Horst-Schrivers, A.N.A.; Links, T.P.; Kerstens, M.N. Incidence of pheochromocytoma and sympathetic paraganglioma in the Netherlands: A nationwide study and systematic review. *Eur. J. Intern. Med.* **2018**, *51*, 68–73. [CrossRef]
3. Lenders, J.W.; Eisenhofer, G.; Mannelli, M.; Pacak, K. Phaeochromocytoma. *Lancet* **2005**, *366*, 665–675. [CrossRef]
4. Y-Hassan, S.; Falhammar, H. Cardiovascular Manifestations and Complications of Pheochromocytomas and Paragangliomas. *J. Clin. Med.* **2020**, *9*, 2435. [CrossRef]
5. van Berkel, A.; Lenders, J.W.; Timmers, H.J. Diagnosis of endocrine disease: Biochemical diagnosis of phaeochromocytoma and paraganglioma. *Eur. J. Endocrinol.* **2014**, *170*, R109–R119. [CrossRef]
6. Heavner, M.G.; Krane, L.S.; Winters, S.M.; Mirzazadeh, M. Pheochromocytoma diagnosed pathologically with previous negative serum markers. *J. Surg. Oncol.* **2015**, *112*, 492–495. [CrossRef]
7. Kota, S.K.; Kota, S.K.; Panda, S.; Modi, K.D. Pheochromocytoma: An uncommon presentation of an asymptomatic and biochemically silent adrenal incidentaloma. *Malays. J. Med. Sci.* **2012**, *19*, 86–91. [PubMed]
8. Ozkaya, M.; Yuzbasioglu, M.F.; Bulbuloglu, E.; Bakaris, S.; Oksuz, H.; Gisi, K.; Onder, A. Incidental pheochromocytoma presenting with sublaboratory findings in asymptomatic surrenal masses: A case report. *Cases J.* **2008**, *1*, 10. [CrossRef]
9. Parasiliti-Caprino, M.; Lucatello, B.; Lopez, C.; Burrello, J.; Maletta, F.; Mistrangelo, M.; Migliore, E.; Tassone, F.; La Grotta, A.; Pia, A.; et al. Predictors of recurrence of pheochromocytoma and paraganglioma: A multicenter study in Piedmont, Italy. *Hypertens. Res.* **2020**, *43*, 500–510. [CrossRef]
10. Neary, N.M.; King, K.S.; Pacak, K. Drugs and pheochromocytoma—Don't be fooled by every elevated metanephrine. *N. Engl. J. Med.* **2011**, *364*, 2268–2270. [CrossRef]
11. Prinzi, N.; Corti, F.; Torchio, M.; Niger, M.; Antista, M.; Pagani, F.; Beninato, T.; Pulice, I.; Rossi, R.E.; Coppa, J.; et al. Metastatic pheochromocytomas and paragangliomas: Where are we? *Tumori* **2022**. *Online ahead of print*. [CrossRef] [PubMed]
12. Fang, F.; Ding, L.; He, Q.; Liu, M. Preoperative Management of Pheochromocytoma and Paraganglioma. *Front. Endocrinol.* **2020**, *11*, 586795. [CrossRef]
13. Lee, J.A.; Zarnegar, R.; Shen, W.T.; Kebebew, E.; Clark, O.H.; Duh, Q.Y. Adrenal incidentaloma, borderline elevations of urine or plasma metanephrine levels, and the "subclinical" pheochromocytoma. *Arch. Surg.* **2007**, *142*, 870–873; discussion 873–874. [CrossRef]
14. Mantero, F.; Terzolo, M.; Arnaldi, G.; Osella, G.; Masini, A.M.; Alì, A.; Giovagnetti, M.; Opocher, G.; Angeli, A. A survey on adrenal incidentaloma in Italy. Study Group on Adrenal Tumors of the Italian Society of Endocrinology. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 637–644. [CrossRef]
15. Kawashima, A.; Sone, M.; Inagaki, N.; Okamoto, K.; Tsuiki, M.; Izawa, S.; Otsuki, M.; Okamura, S.; Ichijo, T.; Katabami, T.; et al. Pheochromocytoma and paraganglioma with negative results for urinary metanephrines show higher risks for metastatic diseases. *Endocrine* **2021**, *74*, 155–162. [CrossRef]
16. Timmers, H.J.; Pacak, K.; Huynh, T.T.; Abu-Asab, M.; Tsokos, M.; Merino, M.J.; Baysal, B.E.; Adams, K.T.; Eisenhofer, G. Biochemically silent abdominal paragangliomas in patients with mutations in the succinate dehydrogenase subunit B gene. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 4826–4832. [CrossRef]
17. Dreijerink, K.M.A.; Rijken, J.A.; Compaijen, C.J.; Timmers, H.; van der Horst-Schrivers, A.N.A.; van Leeuwaarde, R.S.; van Dam, P.S.; Leemans, C.R.; van Dam, E.; Dickhoff, C.; et al. Biochemically Silent Sympathetic Paraganglioma, Pheochromocytoma, or Metastatic Disease in SDHD Mutation Carriers. *J. Clin. Endocrinol. Metab.* **2019**, *104*, 5421–5426. [CrossRef]
18. Puliani, G.; Sesti, F.; Feola, T.; Di Leo, N.; Polti, G.; Verrico, M.; Modica, R.; Colao, A.; Lenzi, A.; Isidori, A.M.; et al. Natural History and Management of Familial Paraganglioma Syndrome Type 1: Long-Term Data from a Large Family. *J. Clin. Med.* **2020**, *9*, 588. [CrossRef]
19. Neumann, H.P.; Pawlu, C.; Peczkowska, M.; Bausch, B.; McWhinney, S.R.; Muresan, M.; Buchta, M.; Franke, G.; Klisch, J.; Bley, T.A.; et al. Distinct clinical features of paraganglioma syndromes associated with SDHB and SDHD gene mutations. *JAMA* **2004**, *292*, 943–951. [CrossRef]
20. Sesti, F.; Feola, T.; Puliani, G.; Centello, R.; Di Vito, V.; Bagni, O.; Lenzi, A.; Isidori, A.M.; Cantisani, V.; Faggiano, A.; et al. Sunitinib Treatment for Advanced Paraganglioma: Case Report of a Novel SDHD Gene Mutation Variant and Systematic Review of the Literature. *Front. Oncol.* **2021**, *11*, 677983. [CrossRef]
21. Feng, F.; Zhu, Y.; Wang, X.; Wu, Y.; Zhou, W.; Jin, X.; Zhang, R.; Sun, F.; Kasoma, Z.; Shen, Z. Predictive factors for malignant pheochromocytoma: Analysis of 136 patients. *J. Urol.* **2011**, *185*, 1583–1590. [CrossRef]

22. Eisenhofer, G.; Lenders, J.W.; Siegert, G.; Bornstein, S.R.; Friberg, P.; Milosevic, D.; Mannelli, M.; Linehan, W.M.; Adams, K.; Timmers, H.J.; et al. Plasma methoxytyramine: A novel biomarker of metastatic pheochromocytoma and paraganglioma in relation to established risk factors of tumour size, location and SDHB mutation status. *Eur. J. Cancer* **2012**, *48*, 1739–1749. [CrossRef] [PubMed]
23. Kim, J.H.; Moon, H.; Noh, J.; Lee, J.; Kim, S.G. Epidemiology and Prognosis of Pheochromocytoma/Paraganglioma in Korea: A Nationwide Study Based on the National Health Insurance Service. *Endocrinol. Metab.* **2020**, *35*, 157–164. [CrossRef] [PubMed]
24. Falhammar, H.; Kjellman, M.; Calissendorff, J. Initial clinical presentation and spectrum of pheochromocytoma: A study of 94 cases from a single center. *Endocr. Connect.* **2018**, *7*, 186–192. [CrossRef]
25. Guerrero, M.A.; Schreinemakers, J.M.; Vriens, M.R.; Suh, I.; Hwang, J.; Shen, W.T.; Gosnell, J.; Clark, O.H.; Duh, Q.Y. Clinical spectrum of pheochromocytoma. *J. Am. Coll. Surg.* **2009**, *209*, 727–732. [CrossRef]
26. Eisenhofer, G.; Lenders, J.W.; Goldstein, D.S.; Mannelli, M.; Csako, G.; Walther, M.M.; Brouwers, F.M.; Pacak, K. Pheochromocytoma catecholamine phenotypes and prediction of tumor size and location by use of plasma free metanephrines. *Clin. Chem.* **2005**, *51*, 735–744. [CrossRef]
27. Pappachan, J.M.; Tun, N.N.; Arunagirinathan, G.; Sodi, R.; Hanna, F.W. Pheochromocytomas and Hypertension. *Curr. Hypertens. Rep.* **2018**, *20*, 3. [CrossRef]
28. Khatiwada, S.; Agarwal, S.; Kandasamy, D.; Jyotsna, V.P.; Kumar, R.; Kumar Bansal, V.; Pandey, R.M.; Gupta, N.; Tandon, N. Diabetes mellitus in pheochromocytoma and paraganglioma: Prevalence, dynamics of insulin secretion/sensitivity and predictors of remission. *Diabetes Metab. Syndr.* **2020**, *14*, 2169–2175. [CrossRef]
29. Elenkova, A.; Matrozova, J.; Vasilev, V.; Robeva, R.; Zacharieva, S. Prevalence and progression of carbohydrate disorders in patients with pheochromocytoma/paraganglioma: Retrospective single-center study. *Ann. D'endocrinologie* **2020**, *81*, 3–10. [CrossRef] [PubMed]
30. Krumeich, L.N.; Cucchiara, A.J.; Nathanson, K.L.; Kelz, R.R.; Fishbein, L.; Fraker, D.L.; Roses, R.E.; Cohen, D.L.; Wachtel, H. Correlation Between Plasma Catecholamines, Weight, and Diabetes in Pheochromocytoma and Paraganglioma. *J. Clin. Endocrinol. Metab.* **2021**, *106*, e4028–e4038. [CrossRef]
31. Eisenhofer, G.; Goldstein, D.S.; Sullivan, P.; Csako, G.; Brouwers, F.M.; Lai, E.W.; Adams, K.T.; Pacak, K. Biochemical and clinical manifestations of dopamine-producing paragangliomas: Utility of plasma methoxytyramine. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 2068–2075. [CrossRef]
32. Lee, M.S.; Lee, R.; Park, S.H.; Kwon, S.; Park, J.Y.; Lee, S.W.; Lee, S.M.; Moon, J.E. Metanephrine Negative Pheochromocytoma: A rare case report of dopamine-secreting tumor in an adolescent patient with NF1. *Ann. Pediatric Endocrinol. Metab.* **2022**. [CrossRef]
33. Miyamoto, S.; Yoshida, Y.; Ozeki, Y.; Okamoto, M.; Gotoh, K.; Masaki, T.; Nishida, H.; Shibuya, T.; Shin, T.; Daa, T.; et al. Dopamine-Secreting Pheochromocytoma and Paraganglioma. *J. Endocr. Soc.* **2021**, *5*, bvab163. [CrossRef]

Article

The Antiproliferative Activity of High-Dose Somatostatin Analogs in Gastro-Entero-Pancreatic Neuroendocrine Tumors: A Systematic Review and Meta-Analysis

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Abstract: Background: The antiproliferative activity of a high dose of somatostatin analogs (HD-SSA) in treating gastro-entero-pancreatic neuroendocrine neoplasms (GEP-NENs) remains under debate. Methods: A systematic review and proportion meta-analysis were made. The primary endpoint was the efficacy measured as incidence density ratio (IDR) at one year. The secondary endpoints were the disease control rate (DCR) and severe adverse events (SAEs). The heterogeneity (I2), when high (>50%), was interpreted by performing a univariate metaregression analysis, analyzing as covariates: type and design of the study, location (Europe or USA), sample size, grading according to 2017 WHO, the metastatic disease rate, previous therapy including surgery, and quality of the study. Results: A total of 11 studies with 783 patients were included. The IDR was 62 new progressions of 100 patients treated with HD-SSA every one year. The heterogeneity was high. The study's year, type and design, primary tumor, grading, previous treatments, and quality of the studies did not influence the IDR. The IDR was significantly higher in USA centers and studies with more than 50 patients. The IDR was lower when a high rate of metastatic patients was present in the studies. The DCR was 45%. The heterogeneity was high. The DCR was lower in USA studies and in prospective trials. Conclusion: Given the limited efficacy of HD-SSA in preventing the disease progression in unresectable GEP-NENs after failure of standard dose SSA, the use of this therapeutic approach is advisable in selected cases when other antiproliferative treatments are not feasible.

Keywords: neuroendocrine neoplasms; high dose of somatostatin analogs; meta-analysis

1. Introduction

According to the WHO classifications, gastro-entero-pancreatic neuroendocrine neoplasms (GEP NENs) are classified based on tumor morphology (well-differentiated neuroendocrine tumors—NET—vs. poorly differentiated neuroendocrine carcinomas—NEC) and grading, which is usually assessed by Ki67 proliferative index (G1 = Ki67 < 3%, G2 = Ki67 3–20%, G3 = Ki67 > 20%) [1,2]. Disease aggressiveness is affected by several factors, including primary tumor site, grading, stage, tumor burden, somatostatin receptors expression, and metabolic activity (assessed by FDG-PET), which are used for evaluating patient's prognosis and for planning the optimal medical treatment when curative surgery is not feasible due to advanced disease [3–5]. Treatment options for patients with NEN are continuously expanding and include long-acting somatostatin analogs (SSAs), peptide

receptor radionuclide therapy (PRRT), tyrosine kinase inhibitors, mTOR inhibitors, and systemic chemotherapy [6].

Long-acting SSAs octreotide and lanreotide are widely considered effective and well-tolerated first-line treatment for G1-G2 GEP NETs expressing sstr, following the results of the phase-3 randomized controlled trials PROMID and CLARINET [7,8]. There are five types of sstr (sstr1–5) whose activation by native somatostatin or SSAs results in antiproliferative effects on tumor cells via direct and indirect mechanisms. Activation of sstr on tumor cells leads to cell cycle arrest and apoptosis through regulation of MAP kinase and phosphotyrosine phosphatase activity, while indirect mechanisms involve the angiogenesis and growth factor secretion inhibition.

SSAs compare favorably with the other approaches available for the treatment of NETs: indeed, SSAs have an excellent toxicity profile and are well-tolerated by patients (with mild gastrointestinal symptoms which are usually transient), have a convenient administration schedule, can control symptoms in patients with hormone-producing tumors, and have shown to have antiproliferative effect. Standard SSA dose is one single injection every 4 weeks, at the standard doses of 30 mg and 120 mg for octreotide and lanreotide, respectively. While native somatostatin binds all sstr types but type 5 (sstr5) at nanomolar concentrations, both SSAs selectively bind with high affinity type 2 sstr (sstr2), which is preferentially expressed on NETs, and with slightly lower affinity sstr5. Their potential increased antiproliferative activity, when used at higher doses in patients not responding to standard dose SSA, has been investigated over the last two decades by several retrospectives or small prospective studies, which report promising results in terms of disease control rates (widely ranging from 30% to 100%) and median progression-free survival (PFS) value (up to 32 months) [9–11]. However, a less favorable outcome, with a median PFS of 8.4 months, was observed with high-dose octreotide (60 mg/4 weeks) in the control group arm of the NETTER-1 study, which was designed to investigate the efficacy of 177Lu-DOTATATE vs. high dose octreotide in midgut NETs [12]. Similarly, in the recently reported phase-2 CLARINET FORTE trial which, patients receiving doubled dose lanreotide (120 mg/2 weeks) after progressing on the standard dose (120 mg/4 weeks) had a median PFS of 8.3 months and 5.8 months in pancreatic and midgut NETs, respectively [13].

Given the heterogeneity of available data on the antiproliferative activity of high-dose (HD) SSAs, we undertook this systematic review and meta-analysis to assess the current literature regarding the efficacy of increasing octreotide or lanreotide dose in patients with progressive GEP NET after standard dose treatment.

2. Materials and Methods

The manuscript was structured following the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement [14].

2.1. Eligibility Criteria, Information Sources, and Search

All studies fulfilling the following PICOS criteria [15] were considered eligible for the present study:

1. Population (P): patients with unresectable GEP-NENs;
2. Interventions (I): HD-SSA;
3. Comparator (C): none;
4. Outcome (O): Progression-free survival (PFS), disease control rate (DCR), and Severe Adverse Events (AEs);
5. Studies: prospective and retrospective studies.

Studies were included when Kaplan–Meier of PFS was reported. Review articles without original data and case reports were excluded. A systematic review of the literature was conducted following the recommendations for systematic reviews in surgery provided by Goossen et al. [16]. The PubMed databases were searched for eligible articles in the English language without publication date or publication type restriction. The last search was carried out on 27 October 2021. The search was conducted using medical subject

headings (MeSH) combined with the following non-MeSH words. The string search used in MEDLINE/PubMed was: (neuroendocrine tumors [MeSH Terms]) AND (octreotide or lanreotide) OR (somatostatin [MeSH Terms]).

2.2. Study Selection and Data Collection Process

The identified records were screened for title and abstract independently by two investigators (M.R. and G.L.). If the paper was considered eligible, the full-text text was evaluated. Data were extracted from the selected articles using a prefixed electronic form. Extracted data were then compared, and any discrepancies were solved through discussion. Any disagreement regarding inclusion criteria was solved through discussion or consulting the last author (D.C.). The PRISMA flow diagram was reported in Figure 1.
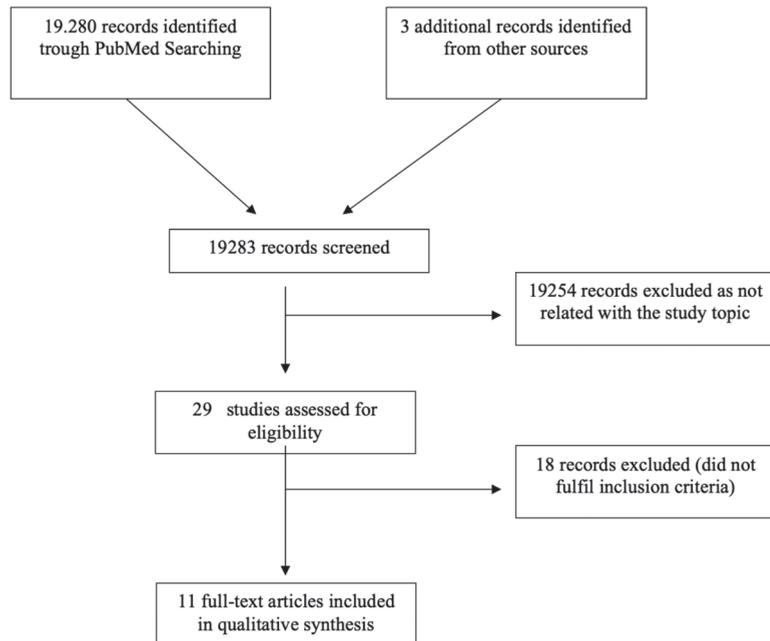
Figure 1. Results of search and reasons for exclusion of papers according to PRISMA statement.

2.3. Data Items, Risk of Bias in Individual Studies, Summary Measures, and Synthesis of Results

The following data were extracted to describe the included studies: year of publication, first author, study type and design, study period, institution and country, study period, number of participants, type, and schedule of SSA. Tumor origin, grading according to the 2017 World Health Organization (WHO) classification [1], metastases, previous treatment with the standard dose of SSA or chemotherapy or surgery of primary tumors were also extracted to evaluate the influence on the outcomes. The quality of studies was assessed with the Risk Of Bias In Non-randomized Studies—of Interventions (ROBINS-I) tool [17]. Incidence density rate (IDR) was used to standardize PFS measurement among the different studies. IDR represents the number of events for at-risk patients per year and makes comparable studies with different observation times. These measures can be assimilated to the hazard rate every year for patients exposed [18]. Thus, the ratio obtained from the IDR incidence density rates can be assimilated to the HR only for the exponential model (constant hazard functions) and the absence of significant differences in the average follow-up duration between the sub-groups [18]. To obtain the crude number of events and observation period from Kaplan–Meier curves, we used dedicated software (GetData Graphical Digitizer@). The results were reported as a pooling proportion (effect size) and

a 95% CI using a random effect model. The meta-analysis was carried out in line with recommendations from the Cochrane Collaboration and Meta-analysis of Observational Studies in Epidemiology guidelines [19,20] and the Mantel–Haenszel random-effects model was used to calculate the effect size [21].

2.4. Risk of Bias across Studies and Additional Analyses

The risk of bias across included studies was measured using the I^2, which describes the variability in point estimates due to heterogeneity rather than sampling error [22]. When I^2 was <50%, the risk of "between-study heterogeneity" was judged as low-moderate; if I^2 was $\geq$50%, the risk of "between-study heterogeneity" was considered high. The meta-regression analysis was performed when heterogeneity was high. The meta-regression was performed using the maximum residual likelihood (REML) approach [23]. The values obtained from metaregression represent the HRs for PFS and the ORs for the DCR and severe AEs, obtained comparing the subgroups. R^2 indicates the heterogeneity explained by the covariate. A *p* value < 0.05 was considered statistically relevant.

The statistical analysis was carried out using dedicated packages for STATA version 14® (StataCorp, College Station, TX, USA).

3. Results

3.1. Study Selection

Article selection process is shown in Figure 1. A total of 19,283 articles were screened, but only 29 studies were evaluated in full-text form. Of these, 18 were excluded because they did not meet inclusion criteria. Finally, only 11 papers [10–13,24–30] were considered suitable for the meta-analysis.

3.2. Study Characteristics and Risk of Bias within Studies

All the papers were published between 1994 and 2021. Eleven studies involving 783 patients were included. There were eight retrospective and three prospective cohorts. The majority of the studies (63.6%) were multicentric and conducted exclusively in European countries. The median sample size of the studies was 54 (range 12–140). The different schedules used are reported in Table 1. The majority of studies (81.8%) have a moderate risk of bias. The other potentially relevant confounding factors are reported in Supplementary Table S1.

Table 1. Characteristics of 11 included studies.

Year	Authors	Study Type	Study Design	Center(s) (Country)	Study Period	Patients Enrolled	Therapy	ROBINS-I
1994	Di Bartolomeo et al. [24].	Retrospective	Multicentric	13 Italian centers	1992–1994	58	1.5 mg daily; 3 mg daily	Moderate
1996	Arnold et al. [25].	Retrospective	Multicentric	49 German centers	1989–1991	103	1.5 mg daily	Moderate
1999	Faiss et al. [26].	Retrospective	Multicentric	3 German centers	Not reported	30	15 mg daily ^	Moderate
2004	Welin et al. [27].	Retrospective	Monocentric	Sweden	Not reported	12	160 mg every 2–4 week °	Moderate
2009	Chadha et al. [28].	Retrospective	Monocentric	USA	2002–2007	30	>30 mg every month §	Moderate
2012	Ferolla et al. [29].	Prospective	Multicentric	Italy	2007–2008	28	30 mg every 3 week §	Moderate
2017	Strosberg et al. [12].	Prospective	Multicentric	41 centers, 8 countries world-wide	2012–2016	113	60 mg every 4 week §	Low

Table 1. *Cont.*

Year	Authors	Study Type	Study Design	Center(s) (Country)	Study Period	Patients Enrolled	Therapy	ROBINS-I
2018	Lau et al. [30].	Retrospective	Monocentric	Canada	2000–2013	65	>30 mg every month [§]	Moderate
2019	Lamberti et al. [10].	Retrospective	Multicentric	Italy	2004–2017	140	180 mg every 4 week [^] or 60 mg every 4 week [§]	Moderate
2021	Pavel et al. [13].	Prospective	Multicentric	25 European centres	2015–2019	51 + 48 [#]	120 mg every 14 days [^]	Low
2021	Diamantopoulos et al. [11].	Retrospective	Monocentric	UK	2003–2017	105	120 mg every 21 days [^]	Moderate

Legend: [^] = Lanreotide; [°] = Octeotride Pamoato: [§] = Octreotide Acetato; [#] = 51 midgut and 48 pancreatic endocrine neoaplasm (panNET); PFS = progression free-survival; DCR = Disease Control Rate; Severe AEs = Severe Adverse Events.

The meta-analytic results are reported in Table 2 and Figures 2–4. The proportion of patients who experienced a disease progression was 62% (53 to 70, 95% CI) per 100 subjects treated every year. Pooled DCR and severe Aes rates were 45% (24 to 64, 95% CI) and 9% (3% to 14%, 95% CI), respectively. All results are affected by high heterogeneity: 96.4%, 98.1%, and 88%, for PFS, DCR, and severe Aes.

Table 2. Results of meta-proportion analysis.

Endpoints	Number of Studies	Effect Size (95% CI)	*p*-Value	Heterogeneity I^2 (%)
PFS	11 [§]	0.62 (0.53 to 0.70)	<0.001 *	96.4
DCR	11 [§]	0.45 (0.24 to 0.64)	<0.001 *	98.1
Severe Aes	11 [§]	0.09 (0.03 to 0.14)	<0.001 *	88

Legend: * = the referent for effect size is the zero value; when *p*-value is <inferior to 0.05, the event is statistically significant; PFS = progression-free survival; DCR = disease control rate; [§] = 11 studies counting 12 cohorts were included because Pavel et al. [13] reported pancreatic and small intestinal NENs results separately.
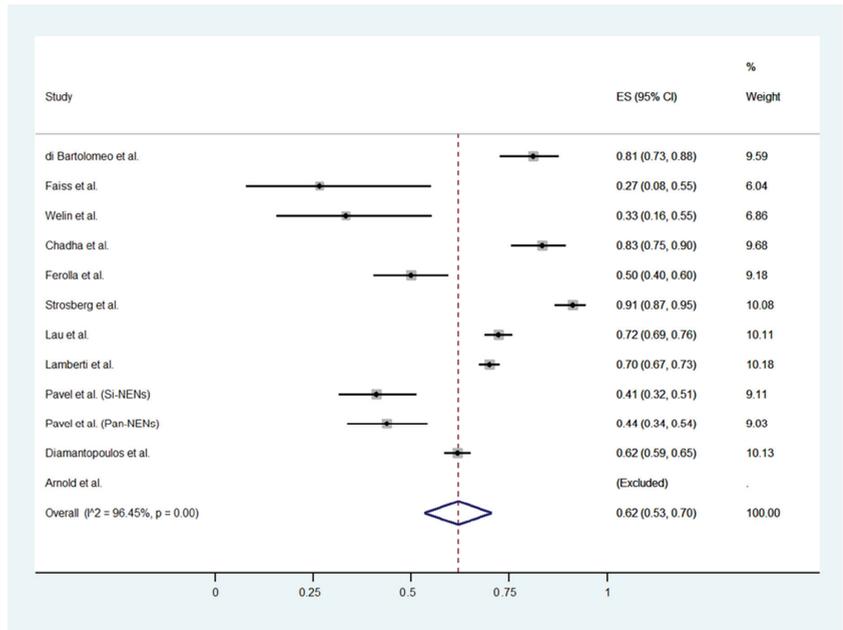
Figure 2. Forest plot for pooled incidence density rate of PFS in patients treated with HD-SSA [10–13,24–30].
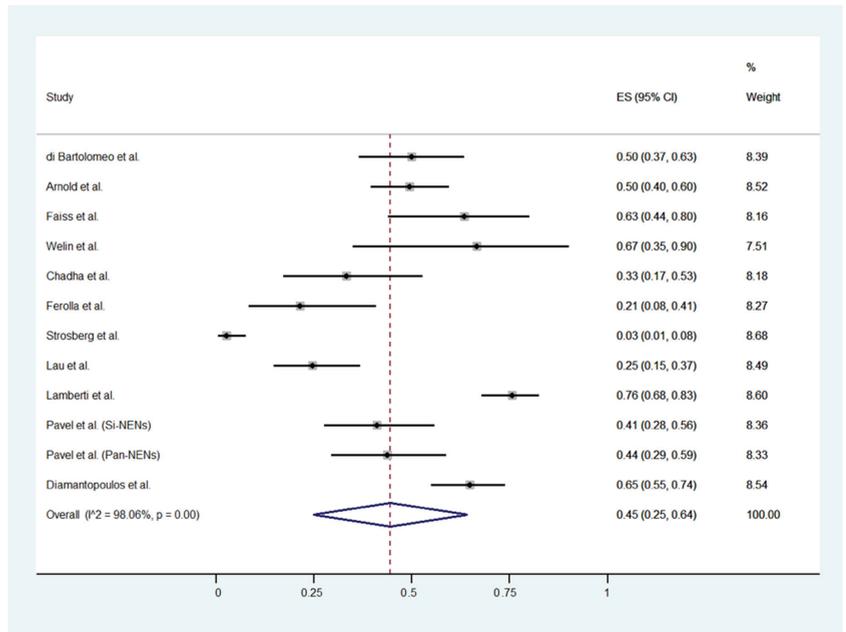
Figure 3. Forest plot for pooled incidence density rate of DCR in patients treated with HD-SSA [10–13,24–30].
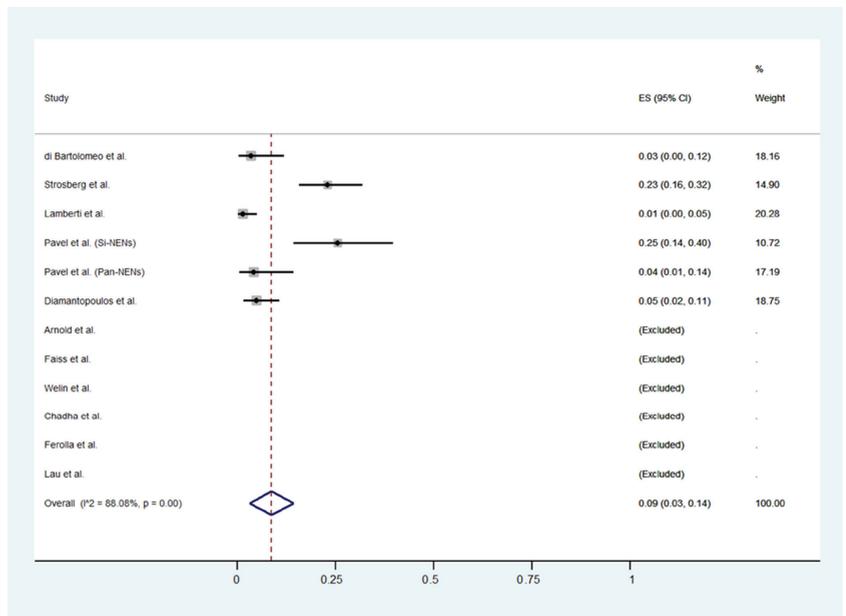
Figure 4. Forest plot for pooled incidence density rate of Aes in patients treated with HD-SSA [10–13,24–30].

At univariate meta-regression analysis, PFS was significantly influenced by three factors (Table 3). The risk of progression was significantly higher in the studies coordinated by USA centers (HR 1.23; 1.03 to 1.45; *p* = 0.021) and when more than 54 patients were enrolled (HR 1.31; 1.04 to 1.64; *p* = 0.023, Figure 5).
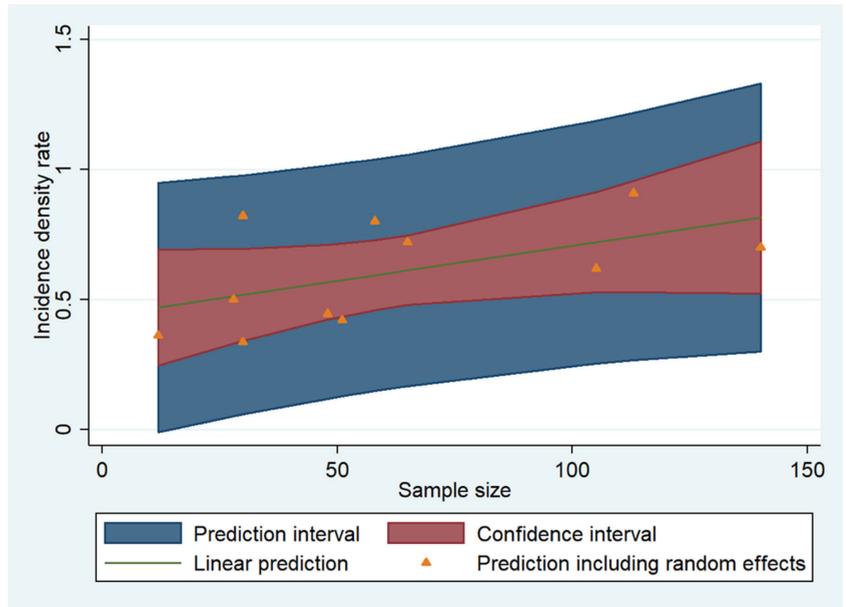
Figure 5. Results of metaregression analysis: the relationship between the incidence density rate and sample size of the studies.

The risk of recurrence was lower in studies with a high rate of metastatic patients (HR 0.35; 0.14 to 0.87; 95% CI). The DCR rate was lower in USA studies (OR 0.76; 0.59 to 0.98; *p* = 0.040) and in prospective trials (0.77; 0.67 to 0.89; *p* < 0.003). No factor explained the heterogeneity of severe Aes.

Table 3. Univariate meta-regression analysis for incidence density ratio, disease control rate, severe adverse events.

| Covariates	Number of Studies	PFS			DCR			Severe Aes		
		HR (95 CI)	R^2 (%)	*p*-Value	OR (95 CI)	R^2 (%)	*p*-Value	OR (95 CI)	R^2 (%)	*p*-Value
Publication year (before vs. after 2000)	11	1.07 (0.77 to 1.50)	0	0.570	0.88 (0.63 to 1.23)	0	0.404	1.08 (0.80 to 1.50)	0	0.570
Study type (retrospective vs. prospective)	11	1.10 (0.87 to 1.39)	5	0.350	0.77; 0.67 to 0.89	16	0.003	1.10 (0.87 to 1.40)	5	0.305
Study design (multicenter vs. uncenter)	11	1.06 (0.76 to 1.49)	0	0.655	0.97 (0.70 to 1.33)	0	0.825	1.06 (0.76 to 1.49)	0	0.655
Study coordinator center (Europe vs. USA)	11	1.23 (1.04 to 1.45)	41	0.023	0.77 (0.67 to 0.89)	60	0.003	1.08 (0.95 to 1.24)	53	0.157
Sample of size (≤ or >median value)	11	1.31 (1.04 to 1.63)	41	0.023	1.10 (0.74 to 1.35)	0	0.990	0.94 (0.72 to 1.24)	0	0.586
Rate of Pan-NENs (increasing)	11	0.91 (0.52 to 1.59)	0	0.109	1.01 (0.58 to 1.80)	0	0.944	0.87 (0.63 to 1.21)	4	0.310
Rate of Si-NENs (increasing)	11	1.05 (0.64 to 1.71)	0	0.839	0.93 (0.61 to 1.44)	0	0.839	1.18 (0.90 to 1.56)	45	0.143
Rate of CR-NENs (increasing)	11	0.26 (0.01 to 7.31)	11	0.372	7.01 (0.57 to 87.5)	25	0.110	0.33 (0.01 to 11.5)	0	0.397
G1 ^ (increasing rate)	6	1.37 (0.56 to 3.32)	0	0.401	0.80 (0.24 to 2.7)	0	0.668	1.45 (0.69 to 3.06)	32	0.212
Metastatic patients (increasing rate)	11	0.36 (0.14 to 0.87)	36	0.029	1.13 (0.34 to 3.73)	0	0.814	1.69 (0.77 to 3.70)	36	0.136
Previous SD of SSA (increasing rate)	11	1.29 (0.78 to 2.12)	3	0.987	1.01 (0.67 to 1.51)	0	0.989	1.42 (0.29 to 6.99)	0	0.570
Previous chemotherapy (increasing rate)	9	0.95 (0.06 to 15.2)	0	0.840	0.98 (0.19 to 4.99)	0	0.980	0.46 (0.01 to 51.73)	0	0.555
Surgery of primary tumors (increasing rate)	10	1.12 (0.43 to 2.86)	0	0.794	0.89 (0.45 to 1.74)	0	0.719	1.01 (0.46 to 2.17)	0	0.995
Risk of bias (low vs. moderate)	12	1.02 (0.72 to 1.44)	0	0.893	1.24 (0.92 to 1.68)	14	0.145	0.87 (0.73 to 1.05)	43	0.118

Legend: PFS = Progression-free survival; DCR = Disease control rate; ADE = adverse events; HR = Hazard Ratio; OR = Odds Ratio; R^2 = % of heterogeneity explained by covariate;
USA = United States of America; Pan-NENs = pancreatic neuroendocrine neoplasms; Si-NENs = small intestinal neuroendocrine neoplasms; CR-NENs = Colo-Rectal neoplasms;
^ = grade according to 2017 WHO classification; SD = Standard Dose; SSA = Somatostatin Analogs.

4. Discussion

SSAs are the mainstay of treatment of well-differentiated GEP-NET since they showed antiproliferative effect [7,8] with a very tolerable safety profile [31,32]. Subsequent treatment lines at progression include PRRT, TKIs, or chemotherapy, all of which have a toxicity profile less favorable than SSAs, with PRRT preferred over other alternatives [33,34]. Non-conventional doses SSA (HD-SSA), achieved by either dose density or dose intensity increase, have been proposed and investigated as a potential treatment option in patients with GEP-NET whose disease progressed on standard dose SSA. Well-differentiated NETs are a heterogeneous group of tumors whose prognosis varies hugely based on baseline clinicopathological variables, including previous evidence of radiological progression, primary organ of origin, Ki67, and extra-hepatic involvement among others, as showed by the highly different median PFS observed in the CLARINET and PROMID studies of lanreotide autogel and octreotide, respectively [7,8], and in the studies investigating the role of HD-SSA (with median PFS ranging from 5 to 30 months). Our meta-analysis showed a relatively high proportion of patients who experience disease progression per year while on HD-SSA, with a discrete rate of DCR as best response and a low incidence of severe adverse events. However, the studies included in the present meta-analysis are highly heterogenous, as captured by an I^2 of approximately 90% and above. To investigate this aspect, a metaregression was performed that showed that DCR is lower in prospective studies than in retrospective ones and in those carried out in the USA compared to those carried out in Europe, with the latter applying also to PFS. An explanation to these findings might be that studies with more rigorous tumor response assessment criteria are more likely to identify and report earlier progressive disease. On the other hand, PFS is shorter in studies with less metastatic patients likely because progressive disease might be more difficult to identify in patients with multiple metastasis, e.g., miliary liver involvement or type III pattern [35]. Furthermore, PFS is shorter for studies with greater sample size, possibly because it tracks with better-conducted, more rigorous studies, with stricter criteria for tumor response evaluation assessment and report of disease progression.

Reviewing the most significant studies, it was found that in the CLARINET FORTE phase II trial of lanreotide 120 mg every 14 days in patients with midgut (N = 79) or pancreatic NET (N = 79), a dramatic decrease in median PFS was observed in tumors with Ki67 >10% as compared to those with a lower proliferation index, in both the midgut (5.5 vs. 8.6 months, respectively) and the pancreatic cohort (2.8 vs. 8.0 months, respectively) [13]. Findings from the CLARINET FORTE trial are in line with the findings of our meta-analysis as it shows that HD-SSA is a feasible treatment option with acceptable PFS outcome only in a subset of patients with pancreatic NET progressing on standard dose SSA, namely those with ki67 $\leq$ 10% as per the post-hoc analysis of the trial. In a retrospective UK series of 105 patients with GEP-NET who each received either lanreotide autogel 120 mg or octreotide 30 mg every 3 weeks, median PFS was 25 months ad it was shorter in patients with PFS < 12 months to previous standard-dose SSA treatment, pancreatic primary, Ki-67 $\geq$ 5% and extrahepatic metastases [11]. However, in this study 58% of patients received HD-SSA because of symptoms progression and 11% because of elevation in serum biomarkers, which could have selected for more indolent disease on the radiological progression side and explain the long PFS observed. Nevertheless, in a large Italian multicenter retrospective study that included 140 patients with GEP-NET who received HD-SSA upon radiological progression to previous treatment, a median PFS of 31 months in the overall cohort was observed [10]. Furthermore, the median PFS was longer when HD-SSA was used as second-line treatment as compared to later lines of treatment, with a trend toward an association with previous standard dose SSA treatment duration, similarly to that observed in the UK series [11].

5. Conclusions

In conclusion, available literature and the results of our meta-analysis suggest that HD-SSA is not the preferred treatment choice in patients with GEP-NET who progressed

on standard-dose SSA because of the short PFS and low DCR reported, especially when compared with other alternatives, such as PRRT [12,33]. This is markedly more evident in studies carried out in the USA, with prospective design, and in patients with metastatic disease. However, a subset of patients with advanced age, whose disease showed indolent behavior, long PFS on standard-dose SSA (>12 months), low Ki67/grading, and low or no extrahepatic metastatic burden, could benefit from HD-SSA treatment as a low-toxicity effective treatment that can preserve quality of life.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11206127/s1, Table S1: Factors potentially influencing incidence density ratio, disease control rate, and severe adverse events.

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References

1. Lloyd, R.V.; Osamura, R.; Kloppel, G.; Rosai, J. *WHO Classification of Tumours of Endocrine Organs*, 4th ed.; IARC Press: Lyon, France, 2017.
2. Klimstra, D.S.; Kloppell, G.; La Rosa, S.; Rindi, G. Classification of neuroendocrine neoplasms of the digestive system. In *WHO Classification of Tumours: Digestive System Tumours*, 5th ed.; WHO Classification of Tumours Editorial Board, Ed.; International Agency for Research on Cancer: Lyon, France, 2019; Volume 16.
3. Panzuto, F.; Merola, E.; Pavel, M.E.; Rinke, A.; Kump, P.; Partelli, S.; Rinzivillo, M.; Rodriguez-Laval, V.; Pape, U.F.; Lipp, R.; et al. Stage IV Gastro-Entero-Pancreatic Neuroendocrine Neoplasms: A Risk Score to Predict Clinical Outcome. *Oncologist* **2017**, *22*, 409–415. [CrossRef]
4. Tarquini, M.; Ambrosio, M.R.; Albertelli, M.; de Souza, P.B.; Gafà, R.; Gagliardi, I.; Carnevale, A.; Franceschetti, P.; Zatelli, M.C. A tool to predict survival in stage IV entero-pancreatic NEN. *J. Endocrinol. Investig.* **2021**, *44*, 1185–1192. [CrossRef] [PubMed]
5. Carideo, L.; Prosperi, D.; Panzuto, F.; Magi, L.; Pratesi, M.S.; Rinzivillo, M.; Annibale, B.; Signore, A. Role of Combined [^{68}Ga]Ga-DOTA-SST Analogues and [^{18}F]FDG PET/CT in the Management of GEP-NENs: A Systematic Review. *J. Clin. Med.* **2019**, *8*, 1032. [CrossRef]
6. Cives, M.; Pelle', E.; Strosberg, J. Emerging Treatment Options for Gastroenteropancreatic Neuroendocrine Tumors. *J. Clin. Med.* **2020**, *9*, 3655. [CrossRef]
7. Rinke, A.; Müller, H.H.; Schade-Brittinger, C.; Klose, K.J.; Barth, P.; Wied, M.; Mayer, C.; Aminossadati, B.; Pape, U.F.; Bläker, M.; et al. Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: A report from the PROMID Study Group. *J. Clin. Oncol.* **2009**, *27*, 4656–4663. [CrossRef] [PubMed]
8. Caplin, M.E.; Pavel, M.; Ćwikła, J.B.; Phan, A.T.; Raderer, M.; Sedláčková, E.; Cadiot, G.; Wolin, E.M.; Capdevila, J.; Wall, L.; et al. Lanreotide in metastatic enteropancreatic neuroendocrine tumours. *N. Engl. J. Med.* **2014**, *371*, 224–233. [CrossRef] [PubMed]
9. Chan, D.L.; Ferone, D.; Albertelli, M.; Pavlakis, N.; Segelov, E.; Singh, S. Escalated-dose somatostatin analogues for antiproliferative effect in GEPNETS: A systematic review. *Endocrine* **2017**, *57*, 366–375. [CrossRef] [PubMed]
10. Lamberti, G.; Faggiano, A.; Brighi, N.; Tafuto, S.; Ibrahim, T.; Brizzi, M.P.; Pusceddu, S.; Albertelli, M.; Massironi, S.; Panzuto, F.; et al. Nonconventional Doses of Somatostatin Analogs in Patients with Progressing Well-Differentiated Neuroendocrine Tumor. *J. Clin. Endocrinol. Metab.* **2020**, *105*, dgz035. [CrossRef]
11. Diamantopoulos, L.N.; Laskaratos, F.M.; Kalligeros, M.; Shah, R.; Navalkissoor, S.; Gnanasegaran, G.; Banks, J.; Smith, J.; Jacobs, B.; Galanopoulos, M.; et al. Antiproliferative Effect of Above-Label Doses of Somatostatin Analogs for the Management of Gastroenteropancreatic Neuroendocrine Tumors. *Neuroendocrinology* **2021**, *111*, 650–659. [CrossRef]
12. Strosberg, J.; El-Haddad, G.; Wolin, E.; Hendifar, A.; Yao, J.; Chasen, B.; Mittra, E.; Kunz, P.L.; Kulke, M.H.; Jacene, H.; et al. Phase 3 trial of 177Lu-dotatate for midgut neuroendocrine tumours. *N. Engl. J. Med.* **2017**, *376*, 125–135. [CrossRef] [PubMed]
13. Pavel, M.; Ćwikła, J.B.; Lombard-Bohas, C.; Borbath, I.; Shah, T.; Pape, U.F.; Capdevila, J.; Panzuto, F.; Truong Thanh, X.M.; Houchard, A.; et al. Efficacy and safety of high-dose lanreotide autogel in patients with progressive pancreatic or midgut neuroendocrine tumours: CLARINET FORTE phase 2 study results. *Eur. J. Cancer* **2021**, *157*, 403–414. [CrossRef] [PubMed]

14. Moher, D.; Liberati, A.; Tetzlaff, J.; Altman, D.G. Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *J. Clin. Epidemiol.* **2009**, *62*, 1006–1012. [CrossRef] [PubMed]
15. Riley, R.D.; Moons, K.G.M.; Snell, K.I.E.; Ensor, J.; Hooft, L.; Altman, D.G.; Hayden, J.; Collins, G.S.; Debray, T.P.A. A guide to systematic review and meta-analysis of prognostic factor studies. *BMJ* **2019**, *364*, k4597. [CrossRef] [PubMed]
16. Goossen, K.; Tenckhoff, S.; Probst, P.; Grummich, K.; Mihaljevic, A.L.; Büchler, M.W.; Diener, M.K. Optimal literature search for systematic reviews in surgery. *Langenbeck's Arch. Surg.* **2018**, *403*, 119–129. [CrossRef]
17. Sterne, J.A.; Hernán, M.A.; Reeves, B.C.; Savović, J.; Berkman, N.D.; Viswanathan, M.; Henry, D.; Altman, D.G.; Ansari, M.T.; Boutron, I.; et al. ROBINS-I: A tool for assessing risk of bias in non-randomized studies of interventions. *BMJ* **2016**, *355*, i4919. [CrossRef]
18. Bender, R.; Beckmann, L. Limitations of the incidence density ratio as approximation of the hazard ratio. *Trials* **2019**, *20*, 485. [CrossRef]
19. Clarke, M.; Horton, R. Bringing it all together: Lancet-Cochrane collaborate on systematic reviews. *Lancet* **2001**, *357*, 1728. [CrossRef]
20. Stroup, D.F.; Berlin, J.A.; Morton, S.C.; Olkin, I.; Williamson, G.D.; Rennie, D.; Moher, D.; Becker, B.J.; Sipe, T.A.; Thacker, S.B. Meta-analysis of observational studies in epidemiology: A proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* **2000**, *283*, 2008–2012. [CrossRef]
21. Mantel, N.; Haenszel, W. Statistical aspects of the analysis of data from retrospective studies of disease. *J. Natl. Cancer Inst.* **1959**, *22*, 719–748. [PubMed]
22. Higgins, J.P.T.; Thompson, S.G. Quantifying heterogeneity in a meta-analysis. *Stat. Med.* **2002**, *21*, 1539–1558. [CrossRef]
23. Thompson, S.G.; Sharp, S.J. Explaining heterogeneity in meta-analysis: A comparison of methods. *Stat. Med.* **1999**, *18*, 2693–2708. [CrossRef]
24. di Bartolomeo, M.; Bajetta, E.; Buzzoni, R.; Mariani, L.; Carnaghi, C.; Somma, L.; Zilembo, N.; di Leo, A. Clinical efficacy of octreotide in the treatment of metastatic neuroendocrine tumors. A study by the Italian Trials in Medical Oncology Group. *Cancer* **1996**, *77*, 402–408. [CrossRef]
25. Arnold, R.; Trautmann, M.E.; Creutzfeldt, W.; Benning, R.; Benning, M.; Neuhaus, C.; Jürgensen, R.; Stein, K.; Schäfer, H.; Bruns, C.; et al. Somatostatin analogue octreotide and inhibition of tumour growth in metastatic endocrine gastroenteropancreatic tumours. *Gut* **1996**, *38*, 430–438. [CrossRef]
26. Faiss, S.; Räth, U.; Mansmann, U.; Caird, D.; Clemens, N.; Riecken, E.O.; Wiedenmann, B. Ultra-high-dose lanreotide treatment in patients with metastatic neuroendocrine gastroenteropancreatic tumors. *Digestion* **1999**, *60*, 469–476. [CrossRef]
27. Welin, S.V.; Janson, E.T.; Sundin, A.; Stridsberg, M.; Lavenius, E.; Granberg, D.; Skogseid, B.; Oberg, K.E.; Eriksson, B.K. High-dose treatment with a long-acting somatostatin analogue in patients with advanced midgut carcinoid tumours. *Eur. J. Endocrinol.* **2004**, *151*, 107–112. [CrossRef]
28. Chadha, M.K.; Lombardo, J.; Mashtare, T.; Wilding, G.E.; Litwin, A.; Raczyk, C.; Gibbs, J.F.; Kuvshinoff, B.; Javle, M.M.; Iyer, R.V. High-dose octreotide acetate for management of gastroenteropancreatic neuroendocrine tumors. *Anticancer Res.* **2009**, *29*, 4127–4130.
29. Ferolla, P.; Faggiano, A.; Grimaldi, F.; Ferone, D.; Scarpelli, G.; Ramundo, V.; Severino, R.; Bellucci, M.C.; Camera, L.M.; Lombardi, G.; et al. Shortened interval of long-acting octreotide administration is effective in patients with well-differentiated neuroendocrine carcinomas in progression on standard doses. *J. Endocrinol. Investig.* **2012**, *35*, 326–331.
30. Lau, S.C.; Abdel-Rahman, O.; Cheung, W.Y. Improved survival with higher doses of octreotide long-acting release in gastroen-teropancreatic neuroendocrine tumors. *Med. Oncol.* **2018**, *35*, 123. [CrossRef] [PubMed]
31. Brighi, N.; Lamberti, G.; Maggio, I.; Manuzzi, L.; Ricci, C.; Casadei, R.; Santini, D.; Mosconi, C.; Lisotti, A.; Ambrosini, V.; et al. Biliary stone disease in patients receiving somatostatin analogs for neuroendocrine neoplasms. A retrospective observational study. *Dig. Liver Dis.* **2019**, *51*, 689–694. [CrossRef] [PubMed]
32. Brighi, N.; Panzuto, F.; Modica, R.; Gelsomino, F.; Albertelli, M.; Pusceddu, S.; Massironi, S.; Lamberti, G.; Rinzivillo, M.; Faggiano, A.; et al. Biliary Stone Disease in Patients with Neuroendocrine Tumors Treated with Somatostatin Analogs: A Multicenter Study. *Oncologist* **2020**, *25*, 259–265. [CrossRef]
33. Ricci, C.; Lamberti, G.; Ingaldi, C.; Mosconi, C.; Pagano, N.; Alberici, L.; Ambrosini, V.; Manuzzi, L.; Monari, F.; Malvi, D.; et al. Treatment of Advanced Gastro-Entero-Pancreatic Neuro-Endocrine Tumors: A Systematic Review and Network Meta-Analysis of Phase III Randomized Controlled Trials. *Cancers* **2021**, *13*, 358. [CrossRef] [PubMed]
34. Spada, F.; Campana, D.; Lamberti, G.; Laudicella, R.; Dellamano, R.; Dellamano, L.; Leeuwenkamp, O.; Baldari, S. [177Lu]Lu-DOTA-TATE versus standard of care in adult patients with gastro-enteropancreatic neuroendocrine tumours (GEP-NETs): A cost-consequence analysis from an Italian hospital perspective. *Eur. J. Nucl. Med. Mol. Imaging* **2022**, *49*, 2037–2048. [CrossRef] [PubMed]
35. Frilling, A.; Li, J.; Malamutmann, E.; Schmid, K.W.; Bockisch, A.; Broelsch, C.E. Treatment of liver metastases from neuroendocrine tumours in relation to the extent of hepatic disease. *Br. J. Surg.* **2009**, *96*, 175–184. [CrossRef] [PubMed]

Article

Bone Loss in Patients with Pancreatic Neuroendocrine Tumors

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Abstract: Background: Pancreatic diseases may affect nutritional status, which is one of the important associated factors of bone health. High prevalence of osteoporosis or osteopenia has been reported in patients with pancreatitis. The bone loss in pancreatic neuroendocrine tumors (PNETs) has not been reported. In this study, we showed the prevalence of bone loss and possible associated factors in PNET patients. Methods: A total of 91 PNET patients were included. Bone status was evaluated based on computed tomography (CT) attenuation (Housfield units, HU): >160 HU, normal bone mineral density; osteopenia, 135 HU $\leq$ CT value $\leq$ 160 HU; osteoporosis, <135 HU. Associated factors for bone loss were identified by logistic regression analyses. Results: The average age was 55.76 years old in PNET patients. The prevalence of osteoporosis and low bone mass was 37.4% and 60.4%, respectively. Higher prevalence of osteoporosis was observed in patients older than 50 years (64.0%). Multivariate logistic analysis showed that age was an associated factor for low bone mass (odds ratio (OR) = 1.13, 95% confidence interval (CI): 1.04–1.22) and osteoporosis (OR = 1.14, 95% CI: 1.03–1.20). Diabetes was also associated with bone loss in PNET patients after adjusting with confounders (OR = 13.56, 95% CI: 1.02–132.4). Conclusions: Our data show that bone loss is common in patients with PNETs. Age and diabetes are associated with bone loss in PNET patients.

Keywords: bone; osteoporosis; pancreatic neuroendocrine tumors

1. Introduction

Osteoporosis is one of the major public health problems and is associated with bone fragility and high fracture risk [1]. It has been reported that patients with gastrointestinal disease (GI) had a high risk of low bone mass or osteoporosis [2]. High prevalence of osteoporosis or osteopenia has been reported in patients with chronic pancreatitis [3–5] and acute pancreatitis [6]. The association between gastric cancer and osteoporosis was also reported [7,8]. Considering neuroendocrine tumors (NET) may have hormone hypersecretion, osteoporosis/osteopenia is also reported in patients with NETs [9]. Pancreatic neuroendocrine tumors (PNETs) represent a rare subgroup of neuroendocrine tumors (NETs) [10]. The estimated annual prevalence of PNETs was 0.48 per year 100,000 persons. Given the improvement in diagnostic techniques, the occurrence of PNETs is increasing [11]. However, the prevalence of bone loss in PNETs has not been clarified, except for a few case reports [12].

Pancreatic diseases may affect nutritional status and is one important associated factor of bone health. PNET may metastasize in bone, increasing the risk of bone loss, break or fracture [13]. In addition, diabetes and obesity are potential risk factors for gastroenteropancreatic NET occurrence [14] and they may also have an impact on bone loss or fracture [15–17]. Moreover, diabetes is associated to a more advanced and progressive

disease [14,18]. However, the role of diabetes in PNET-associated bone loss is still unclear. In the present study, we performed a retrospective analysis to show bone loss in patients with PNETs and possible associated factors.

2. Material and Methods

2.1. Patients

During January 2016 and January 2022, a total of 116 cases of PNETs were found in our institution. Those patients with distal metastasis, without computed tomography (CT) examinations, and who received any treatment, such as surgical resections, radiotherapy, chemotherapy or somatostatin before CT examination were excluded from this study. This retrospective study finally included 91 patients with PNETs. The information of age, gender, history of diabetes mellitus (DM), tumor grade, and tumor size were collected from the medical database. DM was also evaluated by the plasma glucose levels on two separate occasions. Functional PNETs were evaluated by clinical symptoms, such as glucopenia and refractoriness anabrosis. This study was approved by the Ethics Board of the Affiliated Hospital of Nanjing University of Chinese Medicine. Informed consent was obtained from each subjects. This study was performed in accordance with the Declaration of Helsinki.

2.2. PNETs Grade

PNETs grade were divided into three groups based on the ki67 index and mitosis count [19]. Briefly, Grade 1 (G1): Ki-67 $\leq$ 2 and/or mitosis count < 2/10 HPF; G2: Ki-67 index is 3–20 and/or mitosis count is 2–20/10 HPF; G3: Ki-67 index > 20% and/or mitosis count >20 per 10 HPF. G3 tumor was not divided into well-differentiated G3 and pancreatic neuroendocrine carcinoma (PNEC) because of some missing histological information.

2.3. CT Scanning

CT scans were obtained from the multi-detector CT system (GE healthcare, Tokyo, Japan; Philips Brilliance 64, The Netherlands). The CT scanning protocols were as follows: Tube voltage of 120 kV; slice thickness of 2–5 mm and automatic tube current modulation. The images were reconstructed in the workstation using a 0.625 mm section thickness and 0.5 mm increments. The average CT attenuation of the lumbar vertebra (L1-L3) in a region of interest (ROI) through trabecular bone were recorded in Housfield units (HU) for each scan, thus avoiding erosion and sclerosis. The determination of bone attenuation in L1-L3 are shown in Figure 1. Bone loss and osteoporosis were defined based on CT attenuation. Briefly, according to previous defined attenuation thresholds, the cohort was divided into normal bone mineral density (bone attenuation > 160 HU), osteopenia (135 HU $\leq$ bone attenuation $\leq$ 160 HU) and osteoporosis (bone attenuation < 135 HU) [20].
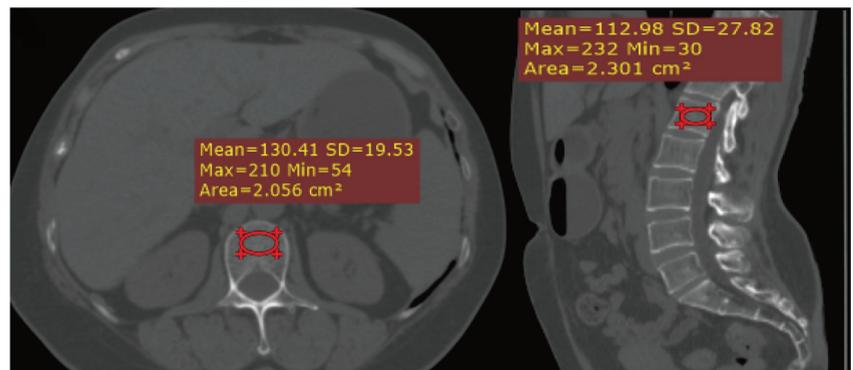

Figure 1. Coronal (**left**) and sagittal (**right**) CT images of lumbar spine (L1) in a 52-year-old women patient with pancreatic neuroendocrine tumors (PNETs).

2.4. Statistical Analysis

The data analyses were performed by SPSS 20.0 (IBM Corp., Armonk, NY, USA). The continuous data was shown as mean ± standard deviation and qualitative data was shown as a number. Correlation analysis was used to show the association between variables. Univariate and multivariate logistic regression analyses were used to identify the associated factor with bone loss. Statistical significance was defined if p value was less than 0.05.

3. Results

3.1. Characteristics of Patients

The characteristics of patients in PNETs are summarized in Table 1. Among 91 patients with PNETs, the mean age was 55.76 years old. There were 35 women and 56 men, respectively. Most of the PNET patients were asymptomatic. Eight patients had functional tumors. The mean CT values of L1-L3 was 158.0 HU. The tumor location was distributed roughly equally among the head-neck (n = 49, 53.8%) and body-tail (n = 42, 46.2%) and the average tumor size was 3.14 cm.

Table 1. Characteristics of patients.

	PNET (n = 91)
Age	55.76 ± 12.97
Gender (women/men)	35/56
Functional tumor	8
Clinical symptoms	
Abdominal pain	29
Weight loss	4
Jaundice	3
Back pain	4
Abdominal mass	0
Glucopenia	6
Asymptomatic	48
Others	8
Post-menopausal status *	27
TNM stage	
T1	27
T2	52
T3	12
N0	76
N1	15
CT value (HU)	158.0 ± 50.11
Location (head-neck/body-tail)	49/42
Size	3.14 ± 1.67
Grade (G1/G2/G3)	20/24/14
CT values (HU)	
>160	36
135–160	21
<135	34

CT: computed tomography; HU: Housfield units; PNETs: pancreatic neuroendocrine tumors (PNETs). * for women.

3.2. The Prevalence of Bone Loss

The prevalence of osteoporosis and bone loss was 37.4% and 60.4% (Table 1), respectively. The prevalence of osteoporosis and low bone mass or osteoporosis in patients with PNETs in men and women are shown in Figure 2. Women tended to have a higher risk of osteoporosis than men (45.7% vs. 32.1%) (Figure 2). Figure 3 showed the prevalence of osteoporosis and low bone mass or osteoporosis in patients older than 50 years. Women tended to have a higher risk of osteoporosis than men (64.0% vs. 36.8%), but no significant difference were observed.
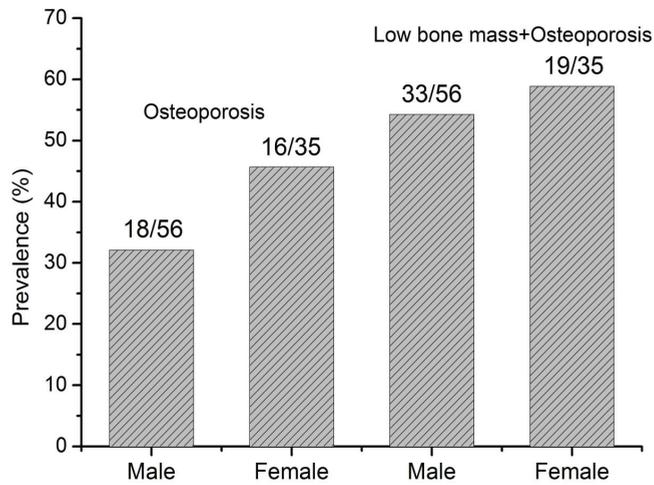
Figure 2. The prevalence of osteoporosis and bone loss (low bone mass + osteoporosis) in patients with pancreatic neuroendocrine tumors (PNETs).
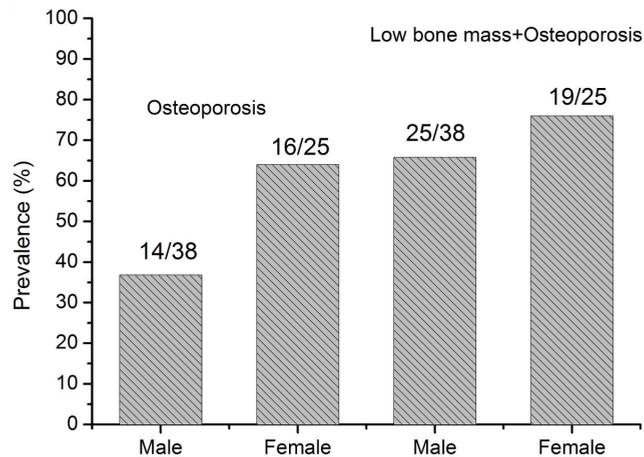
Figure 3. The prevalence of osteoporosis and bone loss (low bone mass + osteoporosis) in old patients (>50 years) with pancreatic neuroendocrine tumors (PNETs).

3.3. Risk Factors for Bone Loss

CT attenuation was negatively correlated with age (r = −0.62, $p < 0.01$) and DM (r = −0.33, $p = 0.01$). However, such an association was not observed between bone CT attenuation and tumor size (r = −0.05, $p = 0.69$) or tumor grade (r = −0.04, $p = 0.74$). Subsequently, logistic regression analysis was used to identify the associated factors. Risk factors for osteoporosis and low bone mass in patients with PNETs are shown in Table 2. The univariate and multivariate logistic regression analyses both showed that age (odds ratio (OR) = 1.11, 95% confidence interval (CI): 1.05–1.17; OR = 1.14, 95% CI: 1.03–1.20) and DM (OR = 10.64, 95% CI: 1.32–118.7; OR = 13.56, 95% CI: 1.02–132.4) were independent risk factors for osteoporosis in patients with PNETs. Univariate and multivariate logistic regression analysis showed that age (OR = 1.12, 95% CI: 1.06–1.18; OR = 1.13, 95% CI: 1.04–1.22) was an independent risk factor for low bone mass.

Table 2. Associations between variables and osteoporosis and low bone mass in pancreatic neuroendocrine tumors (PNETs).

	Osteoporosis		Low Bone Mass	
	Univariate (OR, 95% CI)	Multivariate (OR, 95% CI)	Univariate (OR, 95% CI)	Multivariate (OR, 95% CI)
Age	1.11 (1.05–1.17)	1.14 (1.03–1.20)	1.12 (1.06–1.18)	1.13 (1.04–1.22)
Gender (women vs. men)	1.78 (0.75–4.24)	2.36 (0.68–9.78)	0.83 (0.35–1.94)	0.81 (0.32–2.75)
Location	1.60 (0.53–4.85)	1.74 (0.49–7.69)	1.0 (0.43–2.00)	1.24 (0.43–4.36)
Diabetes mellitus	10.64 (1.32–118.7)	13.56 (1.02–132.4)	/	/
Grade (G3 vs. G1/G2)	0.45 (0.12–1.84)	0.36 (0.04–1.13)	1.28 (0.42–4.54)	0.54 (0.13–4.56)

CI: confidence interval; OR: odds ratio.

4. Discussion

Patients with gastroenteropancreatic-NETs (GEP-NETs) have an increased risk of developing osteopenia and osteoporosis. The bone health of patients with GEP-NETs can also be influenced by hormone hypersecretion, specific microRNAs, nutritional status, or vitamin D deficiency besides the direct effect of bone metastasis [21]. However, the incidence and risk factors of the bone loss in patients with PNETs have not been well recognized. Our data showed that bone loss is common in patients with PNETs (60.4%). Considering that patients with PNETs usually live for a long time, bone health should attract the attention of those patients.

The prevalence of osteoporosis and low bone mass based on quantitative CT (QCT) have been reported in a recent study [22]. For those patients ≥ 50 years, the prevalence of osteoporosis was 28.9% for women, and the prevalence of low bone mass was 42.98% for men and 41.07% for women. Our study showed that the prevalence of osteoporosis and low bone mass in PNETs patients was both higher than the national data from China [22]. The association between DM and PNETs occurrence has been reported [14,23]. Some studies also showed that the occurrence of distant metastasis was higher in PNET patients with DM than those without diabetes [24]. We also showed that DM was a risk factor for osteoporosis or low bone mass in PNETs patients. The association between DM and bone loss or fractures have been widely studied. However, such association was rarely reported in PNET patients. Tumor grades are associated with PNETs treatment and prognosis. However, we did not observe an association between tumor grades and bone loss. One possible explanation is that metastatic PNETs were excluded from our study. Age is the main determined factor for bone loss in the general population. Similar results were observed in our population with PNET, thus suggesting that the role of gonadal status played a critical role in bone health.

Partial or total resections of the pancreas is also associated with osteoporosis [25]. The patients in our study did not receive any treatment, such as surgical resections, radiotherapy, chemotherapy or somatostatin before CT examination. Therefore, the effects of partial or total resections of the pancreas on bone was not studied in our study. We speculated that surgical resection may cause more severe bone loss in PNET patients. The bone health in patients with pancreatic neoplasms is a matter worthy of attention. A healthy lifestyle, such as physical activity, strength training, training to prevent falls, smoking cessation and decreasing alcohol consumption, are important for bone health. Additionally, the American Society of Clinical Oncology (ASCO) suggested that cancer patients should receive a personalize bone mineral density test or take pharmacologic interventions if necessary [26].

The mechanism of how PNET affects bone metabolism are not well clarified. Several related factors have been reported. Briefly, pancreatic diseases may affect the patients' nutritional status because pancreatic juice is important for digestion. Hormone hypersecretion may be another important factor for bone loss in PNET [21]. Functional PNETs may affect

bone metabolism by secreting hormone, such as serotonin (5-HT). NET may also affect the bone by secreting microRNA [20], such as miRNA-201 and miRNA196a.

There are several limitations. First, the sample size was small because of the rarity of PNETs. Our study is a just exploration, and further studies are needed. Second, body mass index may be an associated factor for bone mineral density (BMD). However, we did not collect the data of height and weight. Interestingly, a study showed that QCT-based BMD was not associated with body mass index (BMI) [27]. Therefore, the missing data of BMI may not affect our conclusion. Third, we only showed the prevalence of bone loss, and we did not investigate the possible mechanisms. Fourth, the prevalence of osteoporosis after pancreas resection was not followed up. It would be important to know this data for patient management. Fifth, it would be better to evaluate bone mass by Dual energy X-ray absorption (DXA) or QCT. However, QCT is not routinely performed during abdominal CT scans. Bone CT attenuation can also be used to define bone mass [28]. Finally, we did not have the data of hypogonadism in men, and bone turnover markers, vitamin D status, medical therapies, and other risk factors, such as smoking or corticosteroid use. Prospective studies on this topic are needed.

5. Conclusions

In conclusion, our study showed that the prevalence of osteoporosis and bone loss in patients with PNETs was high. Age and diabetes are the two associated factors with bone loss in PNET patients. Bone health needed attention considering that these patients usually live for a long time.

References

1. Ensrud, K.; Crandall, C. Osteoporosis. *Ann. Intern. Med.* **2017**, *167*, ITC17–ITC32. [CrossRef] [PubMed]
2. Oh, H.J.; Ryu, K.H.; Park, B.J.; Yoon, B.H. Osteoporosis and Osteoporotic Fractures in Gastrointestinal Disease. *J. Bone Metab.* **2018**, *25*, 213–217. [PubMed]
3. Duggan, S.; Smyth, N.; Murphy, A.; Macnaughton, D.; O'Keefe, S.; Conlon, K.C. High prevalence of osteoporosis in patients with chronic pancreatitis: A systematic review and meta-analysis. *Clin. Gastroenterol. Hepatol.* **2014**, *12*, 219–228. [CrossRef]
4. Duggan, S.; Purcell, C.; Kilbane, M.; O'Keane, M.; McKenna, M.; Gaffney, P.; Ridgway, P.F.; Boran, G.; Conlon, K.C. An association between abnormal bone turnover, systemic inflammation, and osteoporosis in patients with chronic pancreatitis: A case-matched study. *Am. J. Gastroenterol.* **2015**, *110*, 336–345. [CrossRef] [PubMed]
5. Hart, P.; Yadav, D.; Li, L.; Appana, S.; Fisher, W.; Fogel, E.; Forsmark, C.E.; Park, W.G.; Pandol, S.; Topazian, M.D.; et al. High Prevalence of Osteopathy in Chronic Pancreatitis: A Cross-sectional Analysis from the PROCEED Study. *Clin. Gastroenterol. Hepatol.* **2021**, *20*, 2005–2013. [CrossRef]
6. Abou Saleh, M.; Alkhayyat, M.; Mansoor, E.; Khoudari, G.; Simons-Linares, C.R.; Vargo, J.; Chahal, P.; Stevens, T. The Risk of Vitamin D Deficiency, Osteoporosis, and Fractures in Acute Pancreatitis. *Pancreas* **2020**, *49*, 629–633. [CrossRef]

7. Jeong, S.M.; Shin, D.W.; Lee, J.E.; Jin, S.M.; Kim, S. Increased Risk of Osteoporosis in Gastric Cancer Survivors Compared to General Population Control: A Study with Representative Korean Population. *Cancer Res. Treat.* **2019**, *51*, 530–537. [CrossRef]
8. Atsumi, Y.; Rino, Y.; Wada, H.; Kitani, Y.; Ozawa, Y.; Aoyama, T.; Oshima, T.; Yukawa, N.; Yoshikawa, T.; Masuda, M. Changes in bone metabolism after gastric cancer surgery in male patients: A prospective observational study. *Gastric Cancer* **2019**, *22*, 237–243. [CrossRef]
9. Hess, G.; Chen, C.; Liu, Z.; Yao, J.; Phan, A.; Hill, J. Clinical burden of illness in patients with neuroendocrine tumors. *Pancreas* **2012**, *41*, 1058–1062. [CrossRef]
10. Dasari, A.; Shen, C.; Halperin, D.; Zhao, B.; Zhou, S.; Xu, Y.; Shih, T.; Yao, J.C. Trends in the Incidence, Prevalence, and Survival Outcomes in Patients With Neuroendocrine Tumors in the United States. *JAMA Oncol.* **2017**, *3*, 1335–1342.
11. Megdanova-Chipeva, V.G.; Lamarca, A.; Backen, A.; McNamara, M.G.; Barriuso, J.; Sergieva, S.; Gocheva, L.; Mansoor, W.; Manoharan, P.; Valle, J.W. Systemic Treatment Selection for Patients with Advanced Pancreatic Neuroendocrine Tumours (PanNETs). *Cancers* **2020**, *12*, 1988. [CrossRef] [PubMed]
12. Byun, J.; Kim, S.; Jeong, H.; Rhee, Y.; Lee, W.; Kang, C. ACTH-producing neuroendocrine tumor of the pancreas: A case report and literature review. Ann. Hepatobiliary. *Pancreat. Surg.* **2017**, *21*, 61–65.
13. Altieri, B.; Di Dato, C.; Martini, C.; Sciammarella, C.; Di Sarno, A.; Colao, A.; Faggiano, A. Bone Metastases in Neuroendocrine Neoplasms: From Pathogenesis to Clinical Management. *Cancers* **2019**, *11*, 1332. [CrossRef]
14. Feola, T.; Puliani, G.; Sesti, F.; Modica, R.; Centello, R.; Minotta, R.; Cannavale, G.; Di Meglio, S.; Di Vito, V.; Lauretta, R.; et al. Risk factors for gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs): A three-centric case-control study. *J. Endocrinol. Investig.* **2022**, *45*, 849–857. [CrossRef] [PubMed]
15. Leslie, W.D.; Morin, S.N.; Majumdar, S.R.; Lix, L.M. Effects of obesity and diabetes on rate of bone density loss. *Osteoporos. Int.* **2018**, *29*, 61–67. [CrossRef] [PubMed]
16. Bai, J.; Gao, Q.; Wang, C.; Dai, J. Diabetes mellitus and risk of low-energy fracture: A meta-analysis. *Aging Clin. Exp. Res.* **2020**, *32*, 2173–2186.
17. Koromani, F.; Oei, L.; Shevroja, E.; Trajanoska, K.; Schoufour, J.; Muka, T.; Franco, O.H.; Ikram, M.A.; Zillikens, M.C.; Uitterlinden, A.G.; et al. Vertebral Fractures in Individuals With Type 2 Diabetes: More Than Skeletal Complications Alone. *Diabetes Care* **2020**, *43*, 137–144.
18. Muscogiuri, G.; Altieri, B.; Albertelli, M.; Dotto, A.; Modica, R.; Barrea, L.; Fanciulli, G.; Feola, T.; Baldelli, R.; Ruggeri, R.M.; et al. Epidemiology of pancreatic neuroendocrine neoplasms: A gender perspective. *Endocrine* **2020**, *69*, 441–450. [CrossRef]
19. Guilmette, J.M.; Nosé, V. Neoplasms of the Neuroendocrine Pancreas: An Update in the Classification, Definition, and Molecular Genetic Advances. *Adv. Anat. Pathol.* **2019**, *26*, 13–30.
20. Rebello, D.; Anjelly, D.; Grand, D.J.; Machan, J.T.; Beland, M.D.; Furman, M.S.; Shapiro, J.; LeLeiko, N.; Sands, B.E.; Mallette, M.; et al. Opportunistic screening for bone disease using abdominal CT scans obtained for other reasons in newly diagnosed IBD patients. *Osteoporos. Int.* **2018**, *29*, 1359–1366. [CrossRef]
21. Altieri, B.; Di Dato, C.; Modica, R.; Bottiglieri, F.; Di Sarno, A.; Pittaway, J.; Martini, C.; Faggiano, A.; Colao, A. Bone Metabolism and Vitamin D Implication in Gastroenteropancreatic Neuroendocrine Tumors. *Nutrients* **2020**, *12*, 1021. [CrossRef] [PubMed]
22. Cheng, X.; Zhao, K.; Zha, X.; Du, X.; Li, Y.; Chen, S.; Wu, Y.; Li, S.; Lu, Y.; Zhang, Y.; et al. Opportunistic Screening Using Low-Dose CT and the Prevalence of Osteoporosis in China: A Nationwide, Multicenter Study. *J. Bone Miner. Res.* **2021**, *36*, 427–435.
23. Halfdanarson, T.R.; Bamlet, W.R.; McWilliams, R.R.; Hobday, T.J.; Burch, P.A.; Rabe, K.G.; Petersen, G.M. Risk factors for pancreatic neuroendocrine tumors (PNETs): A clinic-based case-control study. *Pancreas* **2014**, *43*, 1219–1222. [CrossRef] [PubMed]
24. Fan, Z.; Gong, Y.; Huang, Q.; Yang, C.; Cheng, H.; Jin, K.; Fan, K.; Ni, Q.; Yu, X.; Luo, G.; et al. Diabetes Is Associated With the Metastasis of Pancreatic Neuroendocrine Tumors. *Pancreas* **2020**, *49*, 751–756. [CrossRef] [PubMed]
25. Dammann, H.; Kruse, H.; Kuhlencordt, F.; Montz, R.; Schreiber, H. Resections of the pancreas: Their effects on bone and calcium metabolism. *Z. Gastroenterol.* **1977**, *15*, 577–585.
26. Shapiro, C.; Van Poznak, C.; Lacchetti, C.; Kirshner, J.; Eastell, R.; Gagel, R.; Smith, S.; Edwards, B.J.; Frank, E.; Lyman, G.H.; et al. Management of Osteoporosis in Survivors of Adult Cancers With Nonmetastatic Disease: ASCO Clinical Practice Guideline. *J. Clin. Oncol.* **2019**, *37*, 2916–2946. [CrossRef] [PubMed]
27. Wang, L.; Ran, L.; Zha, X.; Zhao, K.; Yang, Y.; Shuang, Q.; Liu, Y.; Hind, K.; Cheng, X.; Blake, G.M. Adjustment of DXA BMD measurements for anthropometric factors and its impact on the diagnosis of osteoporosis. *Arch. Osteoporos.* **2020**, *15*, 155. [CrossRef] [PubMed]
28. Pickhardt, P.; Pooler, B.; Lauder, T.; del Rio, A.; Bruce, R.; Binkley, N. Opportunistic screening for osteoporosis using abdominal computed tomography scans obtained for other indications. *Ann. Intern. Med.* **2013**, *158*, 588–595. [PubMed]

Article

Role of Selected Circulating Tumor Biomarkers in Patients with Skeletal Metastatic Pancreatic Neuroendocrine Neoplasms

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Abstract: We investigated the diagnostic capacity of selected circulating biomarkers (CBMs) for the early detection of bone metastasis (BMets) in patients with pancreatic neuroendocrine neoplasms (PanNENs). A total of 115 patients with PanNENs and 40 controls were enrolled. We measured the serum levels of ferritin, cytokeratin 18 (CY18), CA19-9, CA125, AFP, CEA, and beta-2 microglobulin (B2M). A total of eight PanNEN patients developed BMets, and one hundred seven remained BMets-free. We observed a significantly higher level of CA125 and CY18 in BMets patients vs. non-BMets patients ($p = 0.01$ and $p = 0.04$, respectively). CA125, CY18, and B2M area under receiver operator characteristic (AUROC) analyses differentiated both patients groups; CA125 area under the curve (AUC) 0.77, $p < 0.01$; CY18 AUC data were 0.72, $p = 0.03$, and B2M AUC 0.67, $p = 0.02$. On the basis of CBM metrics in both subgroups, we reached a sensitivity/specificity for CA125 of 75/76%; for CY18 of 75/69%, for B2M of 100/50%, for CA125, and the CY18 combination of 93/90%, respectively. According to current results, CA125 and CY18 seem to have the potential capacity as fair biomarkers for BMets detection, despite the small number of cases. Further studies are warranted in the larger PanNEN patient group.

Keywords: pancreatic neuroendocrine neoplasms; bone metastasis; biomarker

1. Introduction

Neuroendocrine neoplasms (NENs) of the pancreas constitute about 30% of all gastro–entero–pancreatic neuroendocrine neoplasms (GEP-NENs) and 1–2% of all pancreatic tumors [1]. These tumors can be functional pancreatic neuroendocrine neoplasms (F-PanNENs) or non-functional pancreatic neuroendocrine neoplasms (NF-PanNENs) (60–90%). According to the 5th edition of the World Health Organization (WHO) gastrointestinal system classification (2019), these neoplasms are divided into well-differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs). Additionally, these PanNETs are classified into three subtypes based on the grade of their histological maturity; NET G1—high grade, NET G2—intermediate grade, and NET G3—low grade (according to the number of figures of division and the proliferation index Ki-67).

Over one-third of patients with pancreatic NENs (PanNENs) present with metastatic disease at diagnosis [2]. The 5-year survival rate of PanNENs, for the most part, is associated with distant metastasis [3]. Metastases are present mainly in the liver; however, bone metastases are detected in less than 15% of all NEN patients [4] and in only 4% of pancreatic NEN (PanNEN) patients [4,5]. Bone metastases may be asymptomatic and incidentally detected; therefore, both functional imaging, such as [68Ga]Ga-somatostatin analog (SSA) positron emission tomography (PET)/computed tomography (CT)/[18F]F-FDG PET/CT, and anatomical scans, such as CT/magnetic resonance imaging (MRI) are needed to assess

the disease status of PanNEN [6]. The asymptomatic nature of bone metastases results in an underestimation of the incidence of real bone metastases in NEN patients. The most common symptoms of bone metastases are pain, pathological fractures, and metastatic spinal cord compression. They can lead to malignant hypercalcemia and worsened quality of life [7].

Metastatic disease is always connected with a limited prognosis [8]. Therefore it is essential to find new markers that can predict the probability of metastasis and improve the clinical outcome with accurate treatment. Early detection techniques and treatments for bone metastases, such as bisphosphonate, denosumab, as well as radiation therapy, can significantly reduce the risk of spinal cord compression and pathological fractures, mitigate pain, and thus improve quality of life [9].

Some studies suggest that the presence of certain circulating biomarkers can be useful in the early diagnosis/detection of bone metastases [9,10]. To diagnose bone metastases in PanNEN patients, circulating biomarkers, including ferritin, cytokeratin 18 (CK18), CA19-9, CA125, AFP, CEA, and B2M, were evaluated.

Ferritin is a globular protein, which is synthesized in the liver, spleen, and numerous other body tissues and represents total iron storage in the body. Ferritin can play a role in the angiogenesis, proliferation, and immunosuppression of cancer cells [11]. Unfortunately, the higher level of ferritin correlate with increased disease aggressiveness and worse response to treatment. Additionally, ferritin, through the immune system expression of tumor-associated macrophages, leads to an elevated risk of tumor progression and resistance to treatment [12,13]. In the case of cancer, a higher level of ferritin can also indicate residual neoplastic tissue [14]). CK18 is a structural protein involved in regulating cell growth, apoptosis, mitosis, cancer-related signaling, motility, and many other important processes [15]. It is widely expressed in epithelial tissues of many organs (kidneys, lungs, liver, pancreas, gastrointestinal tract, or mammary gland). Moreover, it is continuously expressed in various cancer tissues and is considered a marker of apoptosis [15,16]. Progression of epithelial tumors is associated with cell apoptosis and increased serum CK18 levels. Studies showed elevated circulating caspase-cleaved cytokeratin 18 in colorectal cancer with liver metastases and hepatocellular carcinoma (HCC) patients. However, in colorectal cancer, levels were significantly higher in patients with higher tumor load and correlated with metastatic volume [16]. Another study suggests that HCC releases CK18 via apoptosis, and HCC patients with low serum CK18 levels have a longer rate of survival [17].

CA19-9 is a cell-surface glycoprotein complex produced by human pancreatic, biliary ductal, gastric, and colon cells. The increased level of CA19-9 may occur in several benign gastrointestinal diseases, but the plasmatic level is severely elevated in pancreatic, biliary, and gastrointestinal cancers [18]. CEA is a non-specific serum marker that functions as a prognostic factor and may monitor the therapy of many neoplasms, such as gastrointestinal carcinomas and lung, breast, pancreatic, and colorectal cancers. The constant increase in CEA levels is usually associated with disease progression, local or distant recurrence.

CA19-9 and CEA are the neoplastic markers assessed mainly in pancreatic cancer (they are increased in 75–85% of pancreatic cancer). CEA sensitivity is far superior to that of CA19-9; however, an increased concentration of CA19-9 is a poor prognostic factor [19]).

CA125 comes under the mucin family of proteins and is a serum tumor marker for multiple cancers, such as ovarian, endometrial, pancreatic, or bladder. It is used to detect the recurrence of the disease, the response to the treatment, and to differentiate malignant and benign lesions [20]. Recent data showed that the serum level of CA125 also correlates with survival in lung cancer [21]. CA125 is expressed on the cell membrane and is unable to penetrate the blood. The membrane damage caused by a.o. inflammation may lead to the elevation of serum CA125 levels [22].

AFP is a glycoprotein produced during embryonic development. In non-pregnant adults, it is present in low serum concentrations, which may be increased in patients with liver, testis, or ovarian cancer. The determination of this marker is important in the management of patients with suspected or diagnosed cancer of the liver, testis, or

ovary [23,24]. B2M is a small molecular weight protein ordinarily present on the surface of all nucleated cells, and it forms the light chain in the human leukocyte antigen [25]. Membrane B2M performs multiple immune functions, while serum B2M is a marker of disease severity in renal injury, infections, amyloidosis, aging-related diseases, and lymphoproliferative disorders [26].

This study aimed to assess the efficacy of various circulating biomarkers in the detection of bone metastases in patients with PanNENs. The early detection of bone metastases is crucial to prevent pathological fractures and physical disability in patients with PanNENs and improves prognosis and quality of life. In case of elevated circulating biomarkers levels, the diagnostic procedure and treatment protocol should be changed: shorter intervals between clinical check-ups and imaging scans and more aggressive treatment at earlier stages of the disease.

2. Materials and Methods

2.1. Study Participants

This study group comprised 115 patients with PanNEN, while the control group consisted of 40 healthy volunteers. The mean age (and range) of the patients in this study group was 53 (19–79), and 50 (25–78) in the control group. The controls were healthy volunteers recruited from the hospital and outpatient clinic personnel. The main inclusion criterion for the patient's group was confirmed histopathological diagnosis of PanNENs according to the WHOs 2019 classification and the American Joint Committee on Cancer/Union for International Cancer Control's 2017 type and signed consent to participate in this study. All patients with PanNEN were recruited at the Department of Endocrinology and Neuroendocrine Tumors, Medical University of Silesia, ENETS Neuroendocrine Tumor Center of Excellence.

Exclusion criteria for studied subjects were: age less than 18, pregnancy, renal, liver or heart insufficiency. The local Ethics Committee approved this study. Information on age, sex, body mass index (BMI), level of chromogranin A, 5-hydroxyindole acetic acid, serotonin, grade, clinical stage, and bone metastasis of the patients with PanNEN was assessed through patients' hospital records. The characteristics of the studied groups are presented in Table 1.

Table 1. Clinical characteristics of the study participants.

Variable	Category	PanNEN Patients	Controls
Number	No.	115	40
Age (years)	Mean (range)	53 (19–79)	50 (25–78)
Gender	Males	49 (43%)	9 (23%)
	Females	66 (57%)	31 (77%)
BMI (kg/m^2)	<30	101 (88%)	N/A
	>30	14 (12%)	
Grade	NET G1	52 (45%)	
	NET G2	45 (39%)	N/A
	NET G3	3 (3%)	
	NEC	5 (4%)	
Clinical stage	I	31 (27%)	
	II	26 (23%)	
	III	14 (12%)	N/A
	IV	44 (38%)	
Bone metastases	Yes	8 (7%)	N/A
	No	107 (93%)	

Abbreviations: BMI, body mass index; N/A, not applicable; NEC, neuroendocrine carcinoma; NET, neuroendocrine tumors; No., number of cases; PanNEN, pancreatic neuroendocrine neoplasms.

Radiological images were reviewed by two independent operators with a huge experience with neuroendocrine neoplasm—a specialist in radiology (if using CT) or a specialist in nuclear medicine (if using PET/CT scan). For the detection of bone metastases

in the majority of patients with PanNEN, we performed a functional examination using [^{68}Ga]Ga-DOTATATE PET/CT ([^{18}F]F-FDG PET/CT. This was performed mainly for poorly differentiated pancreatic neuroendocrine carcinoma (PanNEC).

In 8 patients with PanNEN (3 men and 5 women), bone metastases were confirmed: using CT in 3 cases, [^{68}Ga]Ga-DOTATATE PET/CT in 4 cases, and [^{18}F]FDG PET/CT in 1 case.

This study was conducted in accordance with good clinical practice guidelines and the Declaration of Helsinki.

2.2. Circulating Biomarkers Measurement

The levels of selected biomarkers in the blood serum are described below. The peripheral blood samples (5 mL) were taken from all study participants, leaving the blood to clot. Blood samples from PanNEN patients were taken at different disease stages: before (2 cases) or after tumor-specific treatment (6 cases). Then, these samples were spun, and next, serum was put into boxes and kept at −80 °C for further analysis.

Enzyme–Linked–Immunosorbent Assay (ELISA) or Enzyme–Immunoassay (EIA) was performed with commercially available kits: ELISA kits for B2M, CY18, and ferritin, and EIA kits for AFP, CA125, CA19-9, and CEA. All immunoassays were conducted at the local laboratory in the Department of Endocrinology and Pathophysiology in Zabrze, Medical University of Silesia in Katowice, adapting the manual protocols described by the producers.

The following biomarker tests were used:

- For ferritin: FERRITIN ELISA, DiaMetra S.r.l. Headquater, SEGRATE (Mi), Italy (catalog number DKO039); reference ranges were 20–400 ng/mL for men and 6–350 ng/mL for women; intra-assay precision and inter-assay precision were ≤7.5% and ≤6.1%, respectively.
- For CY18: TPS ELISA, iDL Biotech AB, Bromma, Sweden (catalog number 10-212), the measuring range was 10–1200 U/L, the normal range was <80 U/L, and the detection limit was <6 U/L.
- For CA125: CanAg CA125 EIA, Fujirebio Diagnostics AB, Goteburg, Sweden (catalog number 400-10), the measuring range was 1.5–500 U/mL, the reference range was 5–39 U/mL, the detection limit was <1.5 U/mL, and the intra-assay precision and inter-assay precision were 2.9–4.4% and 3.1–4.0%, respectively.
- For AFP: CanAg AFP EIA, Fujirebio Diagnostics AB, Goteburg, Sweden (catalog number 600-10), the measuring range was 0.5–500 μg/L, the reference range was 0.1–10 μg/L, the detection limit was <0.5 μg/L, and the intra-assay precision and inter-assay precision were 1.6–2.0% and 1.4–2.0%, respectively.
- For CEA: CanAg CEA EIA, Fujirebio Diagnostics AB, Goteburg, Sweden (catalog number 401-10), the measuring range was 0.25–75 μg/L. the reference range was 0.5–9.1 μg/L, the detection limit was <0.25 μg/L, and the intra-assay precision and inter-assay precision were 2.1–2.7% and 1.5–2.7%, respectively.
- For CA19-9: CanAg CA19-9 EIA, Fujirebio Diagnostics AB, Goteburg, Sweden (catalog number 120010), the measuring range was 1–240 U/mL, the reference range was 0–25 U/mL, the detection limit of the assay was <1 U/mL, and the intra-assay precision and inter-assay precision were 3.3–4.5% and 6.2–7.0%, respectively;
- For B2M: β$_2$-Microglobulin ELISA, Immunodiagnostic AG, Bensheim, Germany (catalog number K 6210), the reference range was <2.5 mg/L, and the detection limit of the assay was <0.1 mg/L.

2.3. Statistical Analysis

Data were presented as the median and interquartile range. The comparison of circulating biomarkers concentrations between study and control groups and patients with PanNEN with and without bone metastases was performed using a nonparametric, 2-tailed Mann–Whitney U test. To investigate the diagnostic capacity of circulating biomarkers in detecting bone metastases, receiver operating characteristic (ROC) curves were plotted, and

the area under the curve (AUC), sensitivity, and specificity were calculated. The correlation coefficients between circulating biomarkers concentration, age, BMI, and Ki-67 proliferation index were calculated using the Spearman rank correlation test. The significance threshold in all tests was set at a value of ≤0.05. Statistical analysis was performed using Statistica v. 13.36.0 (StatSoft, Kraków, Poland) software.

3. Results

3.1. Patients with Pancreatic Neuroendocrine Neoplasms vs. Controls

We present the demographic and clinical characteristics of the participants recruited for this study (PanNEN patients and controls) in Table 1. One hundred and fifteen PanNEN patients were recruited, comprising 43% males and 57% females. In contrast to the control subject group, where the proportion of women significantly dominated (77.5%). Most patients (93%) were diagnosed with well-differentiated NET: fifty-two patients had NET G1, while forty-five patients had NET G2. Only seven percent of these patients (8/115) had bone metastases. Bone metastases were identified at different time points, but these were always secondary metastases following liver or lymph node metastases. Comparisons of the studied circulating biomarkers in patients with pancreatic neuroendocrine neoplasms and controls are presented in Tables S1 and S2 in the Supplementary Materials. Serum CY18, ferritin, CA19-9, CEA, and B2M concentrations in PanNEN patients were significantly higher than in control individuals ($p < 0.05$). The highest AUROC for differentiating PanNENs from controls (>0.7) had CY18, CA19-9, and ferritin ($p < 0.001$), which indicates they are fair biomarkers for PanNEN diagnosis. CEA and BMG could also differentiate PanNENs from controls ($p < 0.05$), but AUC < 0.6 indicates poor diagnostic markers.

The pattern of bone metastasis and clinical characteristics of the patients with pancreatic neuroendocrine neoplasms are shown in Table 2.

Table 2. Bone metastasis pattern and clinical characteristics of the patients with pancreatic neuroendocrine neoplasms.

ID	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
Sex	Female	Male	Female	Male	Male	Female	Female	Female
Age (year)	64	25	70	33	74	54	42	60
BMI (kg/m^2)	23.23	21.48	20.78	19.32	29.54	17.31	19.71	27.34
Functional status	NF-PNEN	NF-PNEN	NF-PNEN	NF-PNEN	NF-PNEN	F-PNEN	NF-PNEN	NF-PNEN
Ki-67 (%) of primary	1	10	10	3	3	2	50	60
Grade	NET G1	NET G2	NET G2	NET G2	NET G1	NET G1	NEC	NEC
No. of BM lesion	single	multiple	multiple	multiple	single	multiple	single	single
Localisation of BMets	right pubic bone	vertebrae rib sternum	vertebrae humerus	vertebrae	right rib	vertebrae sacrum	right hip bone	right shoulder blade
Method used for detection of BMets	68Ga PET/CT	CT	68Ga PET/CT	CT	68Ga PET/CT	CT	FDG PET/CT	68Ga PET/CT
Time point of BMets occurrence after initial diagnosis (months)	8	41	16	29	5	3	7	1
Pancreatic primary	body	body	tail	tail	body	tail	head	head
Tumor size (mm)	11	16	43	35	10	83	84	36
Previous type of treatment	surgery	SSA PRRT everolimus CHTH	SSA	CHTH	N/A	N/A	Surgery CHTH RTH	CHTH

Abbreviations: BMets, bone metastasis; BMI, body mass index; CHTH, chemotherapy; CT, computed tomography; [^{18}F]FDG PET, 18F-fluorodeoxyglucose Positron Emission Tomography; F-PanNEN, functional pancreatic neuroendocrine neoplasms; ^{68}Ga PET, Gallium Positron Emission Tomography; NEC, neuroendocrine carcinoma; NET, neuroendocrine tumors; NF-PanNEN, non-functional pancreatic neuroendocrine neoplasms; N/A, not applicable; No., number of cases; PRRT, peptide receptor radionuclide therapy; RTH, radiotherapy; SSA, somatostatin analogs.

3.2. Patients with Pancreatic Neuroendocrine Neoplasm, Bone Metastases and Tumor Biomarkers

In the second part of this study, we established circulating biomarkers levels according to the presence or absence of bone metastases (Table 2). Before circulating biomarker measurements, all PanNEN patients displayed normal routine lab tests, including alkaline phosphatase, ALT/AST, calcium, or phosphate levels, and neuroendocrine tumor marker levels (chromogranin A, serotonin and 5-hydroxyindole acetic acid). The medians of two circulating biomarkers (CY18 and CA125) in PanNEN patients with bone metastases ($n = 8$) were significantly increased ($p < 0.05$) versus those without bone metastases ($n = 107$). The circulating CY18 level in bone metastatic patients (174.20 U/L $\pm$ 121.14; 144 [79–288]) was significantly higher ($p = 0.04$) compared to PanNEN patients without bone metastases (94.17 U/L $\pm$ 93.58; 62 [36–120]). The serum CA125 concentration was also elevated ($p = 0.01$) in the first group (36.29 U/mL $\pm$ 51.47; n = 13 [8–50]) compared to the second group (9.65 U/mL $\pm$ 18.16; n = 6 [3–9]) (Figure 1). The concentrations of other assessed circulating biomarkers, including chromogranin A, serotonin, 5-hydroxyindoleacetic acid, as well as proliferative index Ki-67 and primary tumor size, did not differ significantly between these groups ($p > 0.05$) (Table 3).
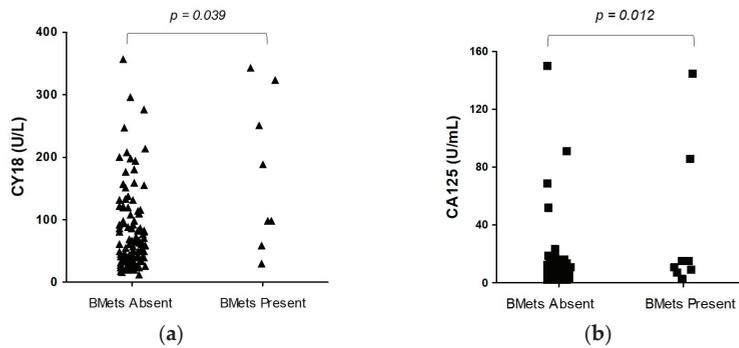

Figure 1. Serum CY18 and CA125 levels in patients with pancreatic neuroendocrine neoplasm (PanNEN): (**a**) Comparison of serum CY18 between PanNEN patients with bone metastasis (BMets) versus PanNEN without BMets ($p = 0.04$); (**b**) Comparison of serum CA125 between PanNEN with BMets versus PanNEN without BMets ($p = 0.01$). Abbreviations: CY18, cytokeratin 18; CA125, cancer antigen 125.

Table 3. The comparison between the clinical characteristics and tumor markers levels in PanNEN patients with (BM-PanNEN patients) and without bone metastasis (non-BM-PanNEN patients) (Mann–Whitney U Test).

Variable	Metastatic PanNEN Patients ($n = 8$) Median [IR]	Non-Metastatic PanNEN Patients ($n = 107$) Median [IR]	*p* Value
Age (years)	57 [38–67]	55 [42–65]	NS
BMI (kg/m²)	21 [20–25]	25 [23–28]	NS
CY18 (U/L)	144 [79–288]	62 [36–120]	0.04
CA125 (U/mL)	13 [8–50]	6 [3–9]	0.01
Ferritin (ng/mL)	129 [47–194]	73 [28–135]	NS
CA19-9 (U/mL)	15 [4–19]	9 [5–16]	NS
AFP (µg/L)	3 [2–12]	3 [2–5]	NS
CEA (µg/L)	2 [1–5]	1 [1–2]	NS
B2M (mg/L)	1 [1–2]	1 [1–2]	NS
CgA (µg/L)	84 [44–678]	45 [27–98]	NS
serotonin (ng/mL)	200 [169–318]	245 [146–372]	NS
5-HIAA (mg/24 h)	3 [3–3]	3 [2–5]	NS
Ki-67 (%)	7 [2–35]	3 [1–5]	NS
Tumor size (mm)	43 [11–83]	27 [16–47]	NS

Abbreviations: ACC, accuracy; AFP, alpha-fetoprotein; B2M, beta-2 microglobulin; CA125, cancer antigen 125; CA19-9, carbohydrate antigens 19-9; CEA, carcinoembryonic antigen; CgA, chromogranin A; CY18, cytokeratin 18; 5-HIAA, 5-hydroxyindoleacetic acid; IR, interquartile range; Ki-67, proliferation index; NS, not significant; PanNEN, pancreatic neuroendocrine neoplasm.

3.3. Diagnostic Accuracy of Tumor Biomarkers

We calculated the AUC and plotted ROC curves to assess the diagnostic value of circulating biomarkers in bone metastases. Given these analyses, three circulating biomarkers (CA125, CY18, and B2M) could differentiate patients with bone metastases from bone-metastases-free subjects ($p < 0.05$). The accuracy of diagnosis in patients with bone metastases was 75% for CA125 compared to 70% for CY18 and 53% for B2M.

3.3.1. Cancer Antigen 125 (CA125)

The median value of CA125 for the PanNEN patients at the time of bone metastatic disease was 13 U/mL and 6 U/l for those of the non-bone metastatic group (Table 3).

The AUC analyses could differentiate PanNEN patients with bone metastases from PanNEN without bone metastases ($p < 0.01$, AUC 0.77 ± 0.09; z score: 2.87, Youden index J: 50%). It should be noted that an AUC of 0.77 would be considered a useful biomarker of bone metastases (Figure 2a). For the cut-off value of 8.87 U/mL for CA125, the specificity/sensitivity was 76/75%, and the accuracy was similarly 75%.

Figure 2. Performance of serum CA125 and CY18 for detecting PanNEN patients with bone metastases (BM-PanNET patients). The receiver operating characteristic (ROC) curves and the area under the curves (AUC) for BM-PanNEN patients versus non-BM-PanNEN patients are displayed: (**a**) Individual ROC curve and AUC for serum CA125 (AUC 0.77, 95% CI 0.59–0.95. $p < 0.01$); (**b**) Individual ROC curve and AUC for serum CY18 (AUC 0.72, 95% CI 0.53–0.91. $p = 0.03$); (**c**) ROC curve and AUC for serum CA125 and CY18 combination (AUC 0.78, 95% CI 0.59–0.95. $p < 0.01$).

3.3.2. Cytokeratin 18 (CY18)

The median value of CY18 was 144 U/L for the PanNEN patients with bone metastases and 62 U/L for those of the non-bone metastatic group (Table 3).

AUC analysis could differentiate PanNEN with bone metastases from PanNEN without bone metastases ($p = 0.03$, AUC 0.72 ± 0.10; z score: 2.22, Youden index J: 44%). It should be noted that an AUC of 0.72 would be considered a fair biomarker of bone metastases (Figure 2b). For the cut-off value of 98.23 U/L for CY18, the accuracy, sensitivity, and specificity were 70%, 75%, and 69%, respectively.

3.3.3. Combination of CY18 and CA125 (multiROC)

Next, we combined the CA125 and CY18 serum levels to construct a further ROC curve. This demonstrated that the serum CA125 and CY18 classifiers had higher accuracy for bone metastases with an AUC similar to CA125 of 0.78 (95% CI 0.59–0.95; Figure 2c). Thus, the combination of CA125 and CY18 in serum was similar to individual CA125 distinguishing between PanNEN with bone metastases and PanNEN without bone metastases (Figure 2c). The sensitivity for the cut-off value of 0.12 was calculated as 63%, and the specificity and accuracy were higher at 93% and 90%, respectively.

The CA125 AUC and CY18 AUC > 0.7 (black curves) indicate they are fair biomarkers for PanNENs with BMets. A maximum AUC = 1 identifies an ideal (perfect) differentiation between these groups. The diagonal red line (AUC = 0.5) corresponds to chance discrimination.

The individual CA125 AUC and combination AUC of CA125 and CY18 were greater than 0.75, which may indicate clinically helpful biomarkers for distinguishing between PanNEN with bone metastases and PanNEN without bone metastases.

3.3.4. Beta-2 Microglobulin (B2M)

The median values of B2M for PanNEN patients with bone metastases and those in the non-bone metastatic group were not significantly different ($p > 0.05$) (Table 3).

Although AUC analyses could differentiate PanNEN with bone metastases from PanNEN without bone metastases ($p = 0.02$), an AUC of 0.67 would be considered a poor biomarker of bone metastases. Youden index J was 50%. The sensitivity and specificity for the cut-off value of 1.16 mg/L were calculated as 100 and 50%, respectively; the accuracy was 53% (Table S3 in Supplementary Materials).

3.3.5. Other Tumor Markers

The median values of other tumor markers (Ferritin, A19-9, AFP, CEA, B2M) for the PanNEN patients with bone metastases and those in the non-bone metastatic group were also not significantly different ($p > 0.05$) (Table 2).

In addition, AUROC analysis of these markers could not differentiate patients with bone metastasis from bone metastasis-free cases (Table S3 in Supplementary Materials).

3.3.6. Neuroendocrine Tumor Markers (Chromogranin A, Serotonin, and 5-Hydroxyinoleacetic Acid)

Based on the AUC and ROC curve analyses of chromogranin A, serotonin, and 5-hydroxyindole acetic acid, we may not differentiate PanNEN with bone metastases from PanNEN without them ($p > 0.05$). The AUC of these tumor markers below 0.6 indicates they are poor predictive markers. These data are presented in Figures S1–S3 in Supplementary Materials.

4. Discussion

The most important factor influencing NEN patients' prognosis is metastasis [3,8]. Most frequently, metastases are located in the liver but can also be found in other organs such as the lungs, brain, or bones [4,5]. The presence of metastasis is always connected with poor prognosis and worse outcomes. Some studies showed that patients with BMets have shorter survival compared to patients with metastasis in other locations [4,9].

We tried to find effective biomarkers that may be useful in the detection of bone metastases in patients with PanNEN. We analyzed potentially valuable proteins such as ferritin, CA19-9, CA125, AFP, CEA, CK18, and B2M. We revealed that levels of three biomarkers (CA125, CY18, and B2M) were significantly higher in patients with metastatic bone disease than those without bone metastases.

Serum cytokeratin (CK) levels are low in healthy individuals. During the process of carcinogenesis, which includes proteolytic degradation in dying cells, abnormal mitosis, and apoptosis, fragments of CKs are released into the blood, and their level is raised [17,27].

As a result, they can be useful as tumor markers and help to predict tumor progression and metastasis formation [27]. Therefore, this study tried to find the correlation between serum CK18 levels and the probability of bone metastases in patients with PanNEN. Cytokeratin 18 exhibits overexpression in many types of cancer originating from epithelial organs [28–30]. A study by Menz A. et al. confirmed the appearance of adenocarcinomas of the lung, pancreas, small bowel, prostate, and cervix uteri [28].

Other investigators showed a higher expression of CK18 in Paget's tumor cells (skin lesions and lymph node metastases). Furthermore, soluble CK18 forms were significantly higher in patients with metastasis compared to non-metastatic disease [31,32].

On the other hand, some studies showed a negative correlation between CK18 concentration and disease advancement (the lower CK18 concentrations were related to lymph node metastasis and poor survival in patients with breast cancer) [33]. A study by Yin B. et al. revealed a negative correlation between serum CK18 level and tumor aggressiveness in prostate cancer [34].

To our knowledge, serum CK18 levels in PanNEN patients with bone metastases were not studied. Our study noted a difference in CK 18 serum levels in patients with and without bone metastases. Patients with bone metastases had a higher level of CK18, so it seems to be clinically useful as a diagnostic factor for bone lesions.

We also tried to find a correlation between the CA125 level and the incidence of bone metastases in patients with PanNEN. Increased CA125 levels can be connected with many malignancies localized in the ovary, breast, liver, lung, pancreas, gastrointestinal tract, uterine, cervix, and endometrium [35]. Its level can also be elevated in healthy individuals such as women in the follicular phase of the menstrual cycle, during pregnancy [35], and in non-malignant conditions such as endometriosis, ovarian cysts, pelvic inflammatory disease, cirrhosis, hepatitis, ascites or heart failure [36–38]. CA125 has been used so far as a marker of ovarian cancer. It has limited sensitivity in detecting ovarian cancer, but it helps monitor response to treatment and detect residual or recurrent disease after therapy. Its level also correlates with staging and tumor size [39–41]. Zhang M. et al. proved that CA125 is significantly elevated not only in ovarian cancer but also in lung and pancreatic cancer and decreased in rectal cancer [42]. In the current study, the level of CA125 was significantly higher in patients with bone metastases versus patients without bone metastatic disease.

Another correlation we observed in this research is the relation between B2M level and the incidence of bone metastases in patients with PanNEN. B2M is involved in many important biological processes, such as the regulation of survival, proliferation, and apoptosis [43,44]. It also stimulates the growth and progression of several cancers or metastasis in cancer cells. Prizment A. et al. pointed out that higher serum B2M is associated with increased colorectal cancer risk. The authors also suggested a significant association between serum B2M and mortality from total, lung, and hematological cancers [45]. The elevated level of B2M is supposed to be a strong indicator of poor prognosis and reduced survival. In prostate cancer, studies found that advanced prostate cancer is connected with an increase in serum levels of B2M [46,47].

Our analysis also tried to find a link between serum levels of common neuroendocrine tumor markers such as chromogranin A (CgA), serotonin, and 5-hydroxy indoleacetic acid (5-HIAA) and bone metastases in PanNETs.

Results of a study by Tomasetti P et al. indicated the diagnostic value of plasma CgA levels in advanced PanNETs with multiple liver metastases [48]. Another study showed higher levels of CgA in metastatic PanNETs compared to localized disease [49]. This effect was also observed in prostate cancer. Patients diagnosed with metastatic castration-resistant prostatic cancer displayed 2–3 times higher levels of CgA compared to those with localized disease [50]. Serotonin and its primary metabolite—5-HIAA is used in the diagnosing and monitoring of carcinoid tumors, a subset of serotonin-secreting neuroendocrine tumors. Studies have shown a potential stimulatory effect of serotonin on cancer cell proliferation, invasion, dissemination, and tumor angiogenesis [51]. Moreover, some research reported that serotonin exerted complex effects on cytokine release from macrophages and mono-

cytes and hence is a crucial factor in controlling the immune microenvironment and may promote tumorigenesis [52].

The analysis performed in this study showed that CgA, serotonin, and 5-HIAA levels did not have the capacity to function as biomarkers for detecting bone metastasis.

Opposite these findings, in an Italian research study by Sara Massironi et al. [53], the median CgA levels were significantly higher in GEP-NEN patients with metastases than those without metastases. In that study, the authors enrolled a total of 181 GEP-NEN patients, including 81 pancreatic NEN, and have shown the significant prognostic relevance of plasma CgA. Similarly, a meta-analysis by Rossi et coauthors [54] revealed that chromogranin A could prevent a diagnosis of recurrence/progression rather than rule it out. It is more reliable when used to monitor disease progression and for the early detection of recurrence after treatment rather than in the diagnostic setting.

In this study, we also tried to find a relationship between other biomarkers (ferritin, CA 19-9, AFP, CEA) and the incidence of bone metastases in patients with PanNEN. The differences between these groups were not statistically significant, so it is possible that in PanNEN, these biomarkers have no utility for bone metastases detection. The useful circulating biomarkers for patients with bone metastases detection were Ca125, CY18, and B2M. They seem to have the diagnostic capacity as fair single biomarkers for the detection of bone metastases. However, the given circulating biomarker measurement performances can not be considered adequate for clinical decision-making. However, more studies on larger groups are required because of the small proportion of patients with bone metastases.

Current research demonstrated a serum panel of biomarkers (CA125 and CY18) to differentiate PanNEN patients with bone metastases from PanNEN patients without bone metastases with good metrics (AUC of 0.78). Indeed, significantly elevated concentrations of these biomarkers in patients with PanNEN may be useful for confirming the clinical suspicion of bone metastases in cases of diagnostic dilemma (difficulties in CT/MRI scan interpretation).

The use of these markers In clinical practice, in our view, could be helpful in the interpretation of unclear bone lesions or screening for further diagnostic workup.

5. Conclusions

It is not possible to draw solid conclusions based on only eight patients with pancreatic neuroendocrine neoplasm with bone metastases. According to current findings, CA125 and CY18 might potentially have the diagnostic capacity as fair single biomarkers for the detection of bone metastases should become despite the small sample size. Further prospective studies are needed in the larger patient group with pancreatic neuroendocrine neoplasm.

6. Study Limitations

First, the total sample of patients with bone metastases was relatively small because PanNEN is rare. Thus, we could not determine a predictive and prognostic value of circulating biomarkers for bone metastases.

Second, the majority of the PanNEN patients were treated before the first presentation of bone metastases.

References

1. Kos-Kudła, B.; Rosiek, V.; Borowska, M.; Bednarczuk, T.; Bolanowski, M.; Chmielik, E.; Ćwikła, J.B.; Foltyn, W.; Gisterek, I.; Handkiewicz-Junak, D.; et al. Pancreatic neuroendocrine neoplasms—Update of the diagnostic and therapeutic guidelines (recommended by the Polish Network of Neuroendocrine Tumours). *Endokrynol. Pol.* **2022**, *73*, 491–548. [CrossRef] [PubMed]
2. Halfdanarson, T.R.; Strosberg, J.R.; Tang, L.; Bellizzi, A.M.; Bergsland, E.K.; O'Dorisio, T.M.; Halperin, D.M.; Fishbein, L.; Eads, J.; Hope, T.A.; et al. The North American Neuroendocrine Tumor Society Consensus Guidelines for Surveillance and Medical Management of Pancreatic Neuroendocrine Tumors. *Pancreas* **2020**, *49*, 863–881. [CrossRef]
3. Komek, H.; Ansal Balci, T.; Can, C. Efficacy of Galium-68 DOTATATE PET/CT in the Detection of Metastasis Rate of Well-Differentiated Gastroenteropancreatic Neuroendocrine Tumors. *Asia Ocean J. Nucl. Med. Biol.* **2019**, *7*, 141–148. [CrossRef] [PubMed]
4. Riihimäki, M.; Hemminki, A.; Sundquist, K.; Sundquist, J.; Hemminki, K. The epidemiology of metastases in neuroendocrine tumors. *Int. J. Cancer* **2016**, *139*, 2679–2686. [CrossRef] [PubMed]
5. Hermans, B.C.M.; de Vos-Geelen, J.; Derks, J.L.; Latten, L.; Liem, I.H.; van der Zwan, J.M.; Speel, E.M.; Dercksen, M.W.; Dingemans, A.C. Unique Metastatic Patterns in Neuroendocrine Neoplasms of Different Primary Origin. *Neuroendocrinology* **2021**, *111*, 1111–1120. [CrossRef]
6. Alexandraki, K.I.; Pizanias, M.; Uri, I.; Thomas, D.; Page, T.; Kolomodi, D.; Low, C.S.; Adesanya, O.; Tsoli, M.; Gross, D.J.; et al. The prognosis and management of neuroendocrine neoplasms-related metastatic bone disease: Lessons from clinical practice. *Endocrine* **2019**, *64*, 690–701. [CrossRef]
7. Garcia-Torralba, E.; Spada, F.; Lim, K.H.J.; Jacobs, T.; Barriuso, J.; Mansoor, W.; McNamara, M.G.; Hubner, R.A.; Manoharan, P.; Fazio, N.; et al. Knowns and unknowns of bone metastases in patients with neuroendocrine neoplasms: A systematic review and meta-analysis. *Cancer Treat. Rev.* **2021**, *94*, 102168. [CrossRef]
8. Rizzo, F.M.; Vesely, C.; Childs, A.; Marafioti, T.; Khan, M.S.; Mandair, D.; Cives, M.; Ensell, L.; Lowe, H.; Akarca, A.U.; et al. Circulating tumour cells and their association with bone metastases in patients with neuroendocrine tumours. *Br. J. Cancer* **2019**, *120*, 294–300. [CrossRef]
9. Kavecansky, J.; Wei, L.; Caronia, L.; Ramirez, M.T.; Bloomston, M.; Shah, M.H. Bone metastases in well-to-moderately differentiated neuroendocrine tumors: A single institutional review from the Ohio State University Medical Center. *Pancreas* **2015**, *44*, 198–203. [CrossRef]
10. Cives, M.; Quaresmini, D.; Rizzo, F.M.; Felici, C.; D'Oronzo, S.; Simone, V.; Silvestris, F. Osteotropism of neuroendocrine tumors: Role of the CXCL12/CXCR4 pathway in promoting EMT in vitro. *Oncotarget* **2017**, *8*, 22534–22549. [CrossRef] [PubMed]
11. Rosiek, V.; Wójcik-Giertuga, M.; Kos-Kudła, B. Serum tumor markers for detection of bone metastases in patients with lung neuroendocrine neoplasms. *Cancer Treat Res Commun.* **2022**, *31*, 100533. [CrossRef] [PubMed]
12. Alkhateeb, A.A.; Connor, J.R. The significance of ferritin in cancer: Anti-oxidation, inflammation and tumorigenesis. *Biochim. Biophys. Acta* **2013**, *1836*, 245–254. [CrossRef] [PubMed]
13. Wang, W.; Knovich, M.A.; Coffman, L.G.; Torti, F.M.; Torti, S.V. Serum ferritin: Past, present and future. *Biochim. Biophys. Acta* **2010**, *1800*, 760–769. [CrossRef]
14. Kernan, K.F.; Carcillo, J.A. Hyperferritinemia and inflammation. *Int. Immunol.* **2017**, *29*, 401–409. [CrossRef]
15. Wang, P.B.; Chen, Y.; Ding, G.R.; Du, H.W.; Fan, H.Y. Keratin 18 induces proliferation, migration, and invasion in gastric cancer via the MAPK signalling pathway. *Clin. Exp. Pharmacol. Physiol.* **2021**, *48*, 147–156. [CrossRef] [PubMed]
16. Singh Bhangu, J.; Macher-Beer, A.; Schimek, V.; Garmroudi, B.; Tamandl, D.; Unger, L.W.; Bachleitner-Hofmann, T.; Oehler, R. Circulating caspase-cleaved cytokeratin 18 correlates with tumour burden and response to therapy in patients with colorectal cancer liver metastasis. *Clin. Chim. Acta* **2023**, *538*, 53–59. [CrossRef]

17. Eguchi, A.; Iwasa, M.; Tamai, Y.; Yamada, M.; Okuno, K.; Shigefuku, R.; Yoshikawa, K.; Tempaku, M.; Sakaguchi, K.; Tanaka, H.; et al. The prognostic potential of fragmented CK18 serum levels in HCC patients reflecting disease progression and overall hepatocyte damage. *Front. Oncol.* **2022**, *12*, 993705. [CrossRef] [PubMed]

18. Lee, T.; Teng, T.Z.J.; Shelat, V.G. Carbohydrate antigen 19-9—Tumor marker: Past, present, and future. *World J. Gastrointest. Surg.* **2020**, *12*, 468–490. [CrossRef]

19. Lech, G.; Słotwiński, R.; Słodkowski, M.; Krasnodębski, I.W. Colorectal cancer tumour markers and biomarkers: Recent therapeutic advances. *World J. Gastroenterol.* **2016**, *22*, 1745–1755. [CrossRef]

20. Yamashita, T.; Higashi, M.; Sugiyama, H.; Morozumi, M.; Momose, S.; Tamaru, J.I. Cancer antigen 125 expression enhances the gemcitabine/cisplatin-resistant tumor microenvironment in bladder cancer. *Am. J. Pathol.* **2022**, *193*, 350–361. [CrossRef]

21. Saad, H.M.; Tourky, G.F.; Al-Kuraishy, H.M.; Al-Gareeb, A.I.; Khattab, A.M.; Elmasry, S.A.; Alsayegh, A.A.; Hakami, Z.H.; Alsulimani, A.; Sabatier, J.M.; et al. The Potential Role of MUC16 (CA125) Biomarker in Lung Cancer: A Magic Biomarker but with Adversity. *Diagnostics* **2022**, *12*, 2985. [CrossRef]

22. Jacobs, I.; Bast, R.C., Jr. The CA 125 tumour-associated antigen: A review of the literature. *Hum. Reprod.* **1989**, *4*, 1–12. [CrossRef]

23. Johnson, P.J. Tumor Markers in Primary malignancies of the liver. In *Tumor Markers: Physiology, Pathobiology, Technology and Clinical Applications*; Dimandis, E.P., Ed.; AACC Press: Washington, DC, USA, 2002; pp. 269–276.

24. Stenman, U.H.; Alfthan, H. Markers for Testicular Cancer. In *Tumor Markers: Physiology, Pathobiology, Technology and Clinical Applications*; Dimandis, E.P., Ed.; AACC Press: Washington, DC, USA, 2002; pp. 351–359.

25. Katzmann, J.A.; Greipp, P.R.; O'Fallon, W.M.; Kyle, R.A. Serum beta 2-microglobulin. *Mayo. Clin. Proc.* **1986**, *61*, 752–753. [CrossRef] [PubMed]

26. Wang, H.; Liu, B.; Wei, J. Beta2-microglobulin(B2M) in cancer immunotherapies: Biological function, resistance and remedy. *Cancer Lett.* **2021**, *517*, 96–104. [CrossRef] [PubMed]

27. Linder, S. Cytokeratin markers come of age. *Tumour. Biol.* **2007**, *28*, 189–195. [CrossRef]

28. Menz, A.; Weitbrecht, T.; Gorbokon, N.; Büscheck, F.; Luebke, A.M.; Kluth, M.; Hube-Magg, C.; Hinsch, A.; Höflmayer, D.; Weidemann, S.; et al. Diagnostic and prognostic impact of cytokeratin 18 expression in human tumors: A tissue microarray study on 11,952 tumors. *Mol. Med.* **2021**, *27*, 16. [CrossRef] [PubMed]

29. Weng, Y.R.; Cui, Y.; Fang, J.Y. Biological functions of cytokeratin 18 in cancer. *Mol. Cancer Res.* **2012**, *10*, 485–493. [CrossRef]

30. Huang, Y.; Yang, L.; Lin, Y.; Chang, X.; Wu, H.; Chen, Y. Prognostic value of non-invasive serum Cytokeratin 18 detection in gastrointestinal cancer: A meta-analysis. *J. Cancer* **2019**, *10*, 4814–4823. [CrossRef]

31. Urano-Takaoka, M.; Sumida, H.; Miyagawa, T.; Awaji, K.; Nagai, K.; Omatsu, J.; Miyake, T.; Sato, S. Serum Cytokeratin 18 as a Metastatic and Therapeutic Marker for Extramammary Paget's Disease. *Acta Derm. Venereol.* **2022**, *102*, adv00636. [CrossRef]

32. Tas, F.; Karabulut, S.; Yildiz, I.; Duranyildiz, D. Clinical significance of serum M30 and M65 levels in patients with breast cancer. *Biomed. Pharmacother.* **2014**, *68*, 1135–1140. [CrossRef]

33. Shi, R.; Wang, C.; Fu, N.; Liu, L.; Zhu, D.; Wei, Z.; Zhang, H.; Xing, J.; Wang, Y. Downregulation of cytokeratin 18 enhances BCRP-mediated multidrug resistance through induction of epithelial-mesenchymal transition and predicts poor prognosis in breast cancer. *Oncol. Rep.* **2019**, *41*, 3015–3026. [CrossRef]

34. Yin, B.; Zhang, M.; Zeng, Y.; Li, Y.; Zhang, C.; Getzenberg, R.H.; Song, Y. Downregulation of cytokeratin 18 is associated with paclitaxel-resistance and tumor aggressiveness in prostate cancer. *Int. J. Oncol.* **2016**, *48*, 1730–1736. [CrossRef]

35. Moss, E.L.; Hollingworth, J.; Reynolds, T.M. The role of CA 125 in clinical practice. *J. Clin. Pathol.* **2005**, *58*, 308–312. [CrossRef]

36. Bast, R.C., Jr.; Xu, F.J.; Yu, Y.H.; Barnhill, S.; Zhang, Z.; Mills, G.B. CA 125: The past and the future. *Int. J. Biol. Markers* **1998**, *13*, 179–187. [CrossRef]

37. Falcão, F.; de Oliveira, F.R.A.; da Silva, M.C.F.C.; Sobral Filho, D.C. Carbohydrate antigen 125: A promising tool for risk stratification in heart diseases. *Biomark. Med.* **2018**, *12*, 367–381. [CrossRef]

38. Chowdhury, M.A.; Xiubin, Z.; Wei, H.; Chenghao, G. Cancer antigen-125 and ICAM-1 are together responsible for ascites in liver cirrhosis. *Clin. Lab.* **2014**, *60*, 653–658. [CrossRef]

39. Wang, Q.; Feng, X.; Liu, X.; Zhu, S. Prognostic Value of Elevated Pre-treatment Serum CA-125 in Epithelial Ovarian Cancer: A Meta-Analysis. *Front. Oncol.* **2022**, *12*, 868061. [CrossRef]

40. Wohlmuth, C.; Djedovic, V.; Kjaer, S.K.; Jensen, A.; Glasspool, R.; Roxburgh, P.; DeFazio, A.; Johnatty, S.E.; Webb, P.M.; Modugno, F.; et al. CA-125 Levels Are Predictive of Survival in Low-Grade Serous Ovarian Cancer-A Multicenter Analysis. *Cancers* **2022**, *14*, 1954. [CrossRef] [PubMed]

41. Kang, K.N.; Koh, E.Y.; Jang, J.Y.; Kim, C.W. Multiple biomarkers are more accurate than a combination of carbohydrate antigen 125 and human epididymis protein 4 for ovarian cancer screening. *Obstet. Gynecol. Sci.* **2022**, *65*, 346–354. [CrossRef] [PubMed]

42. Zhang, M.; Zhang, Y.; Fu, J.; Zhang, L. Serum CA125 levels are decreased in rectal cancer but increased in fibrosis-associated diseases and in most types of cancers. *Prog. Mol. Biol. Transl. Sci.* **2019**, *162*, 241–252. [CrossRef] [PubMed]

43. Li, L.; Dong, M.; Wang, X.G. The Implication and Significance of Beta 2 Microglobulin: A Conservative Multifunctional Regulator. *Chin. Med. J.* **2016**, *129*, 448–455. [CrossRef]

44. Althubiti, M.; Elzubier, M.; Alotaibi, G.S.; Althubaiti, M.A.; Alsadi, H.H.; Alhazmi, Z.A.; Alghamdi, F.; El-Readi, M.Z.; Almaimani, R.; Babakr, A. Beta 2 microglobulin correlates with oxidative stress in elderly. *Exp. Gerontol.* **2021**, *150*, 111359. [CrossRef] [PubMed]

45. Prizment, A.E.; Linabery, A.M.; Lutsey, P.L.; Selvin, E.; Nelson, H.H.; Folsom, A.R.; Church, T.R.; Drake, C.G.; Platz, E.A.; Joshu, C. Circulating Beta-2 Microglobulin and Risk of Cancer: The Atherosclerosis Risk in Communities Study (ARIC). *Cancer Epidemiol. Biomarkers. Prev.* **2016**, *25*, 657–664. [CrossRef]
46. Zhang, Y.X.; Wang, L.; Ji, P.Y.; Zhao, G.G.; Zhong, G.P.; Wang, Z.P. Correlation of serum β2-microglobulin levels with prostate-specific antigen, Gleason score, clinical stage, tumor metastasis and therapy efficacy in prostate cancer. *Arch. Med. Res.* **2013**, *44*, 259–265. [CrossRef] [PubMed]
47. Mink, S.R.; Hodge, A.; Agus, D.B.; Jain, A.; Gross, M.E. Beta-2-microglobulin expression correlates with high-grade prostate cancer and specific defects in androgen signaling. *Prostate* **2010**, *70*, 1201–1210. [CrossRef] [PubMed]
48. Tomassetti, P.; Migliori, M.; Simoni, P.; Casadei, R.; De Iasio, R.; Corinaldesi, R.; Gullo, L. Diagnostic value of plasma chromogranin A in neuroendocrine tumours. *Eur. J. Gastroenterol. Hepatol.* **2001**, *13*, 55–58. [CrossRef]
49. Paik, W.H.; Ryu, J.K.; Song, B.J.; Kim, J.; Park, J.K.; Kim, Y.T.; Yoon, Y.B. Clinical usefulness of plasma chromogranin a in pancreatic neuroendocrine neoplasm. *J. Korean. Med. Sci.* **2013**, *28*, 750–754. [CrossRef]
50. Szarvas, T.; Csizmarik, A.; Fazekas, T.; Hüttl, A.; Nyirády, P.; Hadaschik, B.; Grünwald, V.; Püllen, L.; Jurányi, Z.; Kocsis, Z.; et al. Comprehensive analysis of serum chromogranin A and neuron-specific enolase levels in localized and castration-resistant prostate cancer. *BJU Int.* **2021**, *127*, 44–55. [CrossRef]
51. Sarrouilhe, D.; Clarhaut, J.; Defamie, N.; Mesnil, M. Serotonin and Cancer: What Is the Link? *Curr. Mol. Med.* **2015**, *15*, 62–77. [CrossRef]
52. Herr, N.; Bode, C.; Duerschmied, D. The Effects of Serotonin in Immune Cells. *Front. Cardiovasc. Med.* **2017**, *20*, 48. [CrossRef]
53. Massironi, S.; Rossi, R.E.; Casazza, G.; Conte, D.; Ciafardini, C.; Galeazzi, M.; Peracchi, M. Chromogranin A in diagnosing and monitoring patients with gastroenteropancreatic neuroendocrine neoplasms: A large series from a single institution. *Neuroendocrinology* **2014**, *100*, 240–249. [CrossRef] [PubMed]
54. Rossi, R.E.; Ciafardini, C.; Sciola, V.; Conte, D.; Massironi, S. Chromogranin A in the Follow-up of Gastroenteropancreatic Neuroendocrine Neoplasms: Is It Really Game over? A Systematic Review and Meta-analysis. *Pancreas* **2018**, *47*, 1249–1255. [CrossRef] [PubMed]

Multidisciplinary Approach to the Diagnosis of Occult Primary Neuroendocrine Neoplasm: A Clinical Challenge

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Abstract: Approximately 11% to 14% of subjects with neuroendocrine neoplasms (NENs) have metastatic lesions with unknown primary origin (UPO), with the majority of UPO-NENs found in the small bowel. Herein, we assessed the available literature on UPO-NENs, focusing on clinical presentation and diagnostic techniques to identify the primary site. The identification of the primary tumor is important as it affects the prognosis; however, the clinical presentation can be non-specific in non-functioning forms. In the presence of metastatic disease, the histological sample is fundamental to obtain immunohistochemical markers that might orientate the clinician in the search for the primary tumor through radiology, functional imaging and endoscopic techniques. In summary, multidisciplinary management plays a key role in UPO-NENs, even more than in other NENs. Molecular biology and gene-expression profiling represent areas of great interest which might be developed in the near future for both the diagnosis and the treatment of these neoplasms.

Keywords: neuroendocrine neoplasms; unknown primary tumor; diagnosis; ultrasound endoscopy; capsule endoscopy; double-balloon enteroscopy; treatment; immunohistochemistry; molecular biology

1. Introduction

Neuroendocrine neoplasms (NENs) are rare tumors that originate in diffuse neuroendocrine cells, potentially affecting any organ. NENs encompass a large and heterogenous group of neoplasms characterized by different biological behavior, depending on the clinical and histopathological features and primary site. NENs are classified into well-differentiated G1–G3 NENs and poorly differentiated G3 neuroendocrine carcinomas (NECs), based on their morphological features and proliferation rate. This dichotomous morphological classification reflects underlying differences in terms of genomic characteristics, clinical and biological behavior. We define NENs of unknown primary origin (UPO-NENs) whenever there is a histologically confirmed metastatic disease without an identifiable primary tumor. The metastatic sites associated with UPO-NENs are the liver, followed by the peritoneum, the lymph nodes and, less often, the bones and the lung [1].

While many studies explore differential NEN outcomes according to their origin, only a few studies have evaluated the outcomes of UPO-NENs. An unknown primary site

can be considered a poor prognostic factor, especially for patients with advanced-stage disease [2]. However, these cancers can be stratified into favorable (approximately 20%) and poor (approximately 80%) prognostic groups, based on clinical presentation, host factors, tumor histology, functionality, disease burden, location of metastatic sites and sensitivity to chemoradiation treatment. Patients with UPO-NENs have an overall survival of 6 to 9 months, although the favorable prognostic group may have a median survival of nearly 36 months [3]. Early localization of the primary tumor is of utmost importance to define the patient's management and prognosis [4]. As a matter of fact, prompt identification of the primary tumor site improves the clinical outcome as, according to available evidence [5,6], the resection of the primary tumor also improves survival in in the presence of liver metastases. An Italian retrospective study [6] including 139 liver-metastatic well-differentiated NENs reported that primary tumor resection was an independent positive prognostic factor in multivariate analysis; notably, also in the group of 103 patients with non-resectable liver metastases, the resection of the primary tumor was significantly associated with prolonged survival. In fact, limiting the disease to the liver allows several potentially curative treatment options, including liver resection or liver transplant (in cases of tumors originating from the gastro-entero-pancreatic tract). Furthermore, the resection of the primary tumor reduces the risk of local complication, particularly in the case of small-bowel NENs, such as occlusion, perforation and/or bleeding [6]. Finally, surgery of the primary tumor allows for a biological assessment of the disease and access to potential treatments which require the primary tumor to be clearly identified.

Based on the above observations, in the current review we assessed the available literature on UPO-NENs, focusing on clinical presentation and diagnostic techniques to identify the primary site (radiological/metabolic imaging, endoscopic procedures and molecular pathology), highlighting the need for prompt identification of the primary tumor site and also providing a potential diagnostic algorithm.

2. Materials and Methods

A bibliographical search was performed in PubMed to identify guidelines and the primary literature (retrospective and prospective studies, systematic reviews, case series) published in the last 15 years, using both medical subject heading (MeSH) terms and free-language keywords: neuroendocrine neoplasms; unknown primary tumor; diagnosis; ultrasound endoscopy; capsule endoscopy; double-balloon enteroscopy; treatment; immunohistochemistry; molecular biology. The reference lists from the studies returned by the electronic search were manually searched to identify further relevant reports. Articles published as abstracts were included, whereas non-English-language papers were excluded.

3. Results

A total of 139 records were reviewed and 58 were defined as fulfilling the criteria for final consideration. Figure 1 presents a flow chart showing the process of study selection.

3.1. Epidemiology

NENs represent around 0.5% of all newly diagnosed neoplasms [7]. In recent decades, the incidence of NENs has hugely increased, likely due to improvements in diagnostic techniques and increased disease awareness [2], being approximately 5.86/100,000 per year [8]. The most frequent primary sites are represented by the gastrointestinal/pancreatic tract (62–67%) and lung (22–27%). In well-differentiated tumors, the majority of metastatic sites are found within the liver only [7].

Approximately 11% to 14% of subjects with NENs present metastatic lesions with a UPO, being the majority of UPO-NENs found in the small bowel [9], particularly for well-differentiated forms, followed by the pancreas. Conversely, in poorly differentiated forms, the primary site is generally located in the lung [10]. In 2020, Abdel-Rahman et al. [11] conducted a real-world, population-based study to evaluate the actual incidence and outcome of UPO-NENs. Out of a total of 51,415 recorded cases with NENs, a total of

3550 cases (7%) were diagnosed with UPO-NENs. The authors observed first that the diagnosis of UPO-NENs has increased across the past 4 decades; furthermore, they reported that metastatic small-intestinal NENs appear to have a better prognosis when compared with metastatic UPO-NENs (for both carcinoid tumors and neuroendocrine carcinomas).

PRISMA 2020 flow diagram for new systematic reviews which included searches of databases and registers only
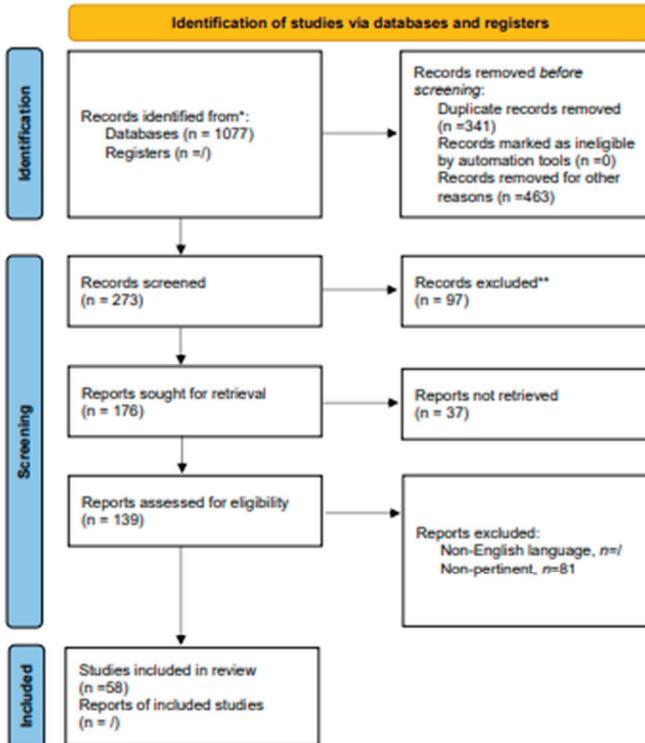
*Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers).

**If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools.

Figure 1. Flow chart showing the process of study selection.

3.2. Clinical Presentation

In the neuroendocrine setting, the majority of symptoms are non-specific and tend to overlap with more common, often gastro-intestinal (GI), conditions, leading to a significant delay in diagnosis. This assumption is particularly true for those cases in which the primary lesion is undetectable thorough conventional imaging techniques [computed tomography (CT) scan, magnetic resonance imaging (MRI)], and the diagnosis of NENs may be, therefore, mistakenly shelved in favor of other endocrine or GI disorders contributing to the aforementioned diagnostic delay.

Clinical features may be related to the tumor's hormonal production (functioning NENs), to the site of the primary tumor or to its metastases (mostly hepatic). Functioning NENs can be responsible for many renowned clinical syndromes (as depicted in Table 1), while non-functioning forms' presentation is often connected to their mass effect.

Table 1. Functioning neuroendocrine neoplasms and their associated clinical syndromes.

Insulinoma	**Whipple's triad** • Hypoglycemia (<50 mg/dL) • Hypoglycemic symptoms (dizziness, sweating, confusion, increased HF) • Symptoms' resolution with glucose ingestion
Gastrinoma	**Zollinger Ellison Syndrome** • Peptic ulcer disease • Diarrhea • Gastro-esophageal reflux disease • Weight loss
VIP-oma	**Verner Morrison Syndrome** • Watery diarrhea • Dehydration • Hyperkalemia
Glucagonoma	• Diarrhea • Glucose intolerance/diabetes • Necrolytic migratory erythema • Weight loss and steathorrhoea • Anemia
Somatostatinoma	• Diarrhea • Weight loss • Diabetes • Gallstones

Midgut/small-intestine NENs (SI-NENs), generally represent the majority of UPO-NENs, which account for 12–22% of all patients diagnosed with NENs [12].

In this scenario, frequent local symptoms include: bowel obstruction or perforation (as a matter of fact, small-bowel NENs are often identified during emergency abdominal surgery), obscure intestinal bleeding without any significative endoscopic finding, unexplained anemia from chronic blood loss or, rarely, obstructive manifestations from vascular compression. Likewise, occult bronchial NENs can be responsible for hemoptysis, dyspnea or recurrent infections due to bronchial obstruction.

The presence of liver metastases can be symptomatic itself by causing abdominal pain (due to liver-capsule stretching or bleeding) or mixed hyperbilirubinemia (as a result of both obstruction and hepatic failure) up to obstructive jaundice. In addition, liver metastases—whether detectable through conventional imaging or not [13]—can be responsible for the development of carcinoid syndrome (CS), a clinical syndrome characterized by flushing, diarrhea and bronchospasm as leading symptoms that can lead to life-threatening complications, such as carcinoid heart disease. The prevalence of CS in patients with NENs has grown significantly in the past decade together with the well-known increase in NENs' incidence: a large American study showed an increase in its incidence from 11% to 19% during the decade 2000–2011 and its association mainly to midgut NENs (40%); moreover, the presence of CS seemed to be linked to a shorter overall survival [14]. In the setting of UPO-NENs, CS can represent the first or the only clinical manifestation (especially if the primary tumor has a small size), but, again, its symptoms can be mistaken for other conditions (including anxiety, irritable bowel syndrome, menopause, allergic asthma) and the presence of liver metastases frequently ends up being an incidental finding. It is, indeed, a common experience that the diagnosis of NENs is generally delayed and patients with small-bowel NENs are often erroneously diagnosed with irritable bowel syndrome or inflammatory bowel disease due to the non-specific clinical presentation.

3.3. Diagnostic Work-Up

Localization of midgut tumors might be challenging due to their usually small size. Early localization of the primary site is a fundamental prerequisite for improving the

patient's management and prolonging survival [4], especially for patients with well-differentiated NENs.

A continuum of investigations to identify the primary tumor is warranted. A multi-modal imaging approach, including CT, MRI, positron emission tomography (PET) and somatostatin receptor scintigraphy (SRS) together with endoscopy, is often necessary for detecting the primary tumor [15,16]. In addition to conventional upper and lower GI endoscopy, more sophisticated techniques, including CT enterography, CT angiography, video capsule endoscopy or double-balloon enteroscopy and endoscopic ultrasonography, may all be combined to shed light on challenging cases [16,17]. In selected cases, whenever all the available diagnostic tools have failed, surgical exploration may be warranted. In this setting, an open exploration is considered to be superior to laparoscopy when the primary site cannot be identified but the data are limited [17,18]. However, despite surgical exploration, the primary site is not found in approximately 13% of the cases [16].

The presence of a functional syndrome might be of help to identify the site of the primary lesion in UPO-NENs. In fact, CS is typically secondary to an NEN located in the small bowel and, in this setting, 5-hydroxyindoleacetic acid (5-HIAA) urine levels should be determined, being the specific biomarker for CS [19]. On the other hand, when a functioning NEN as a gastrinoma is suspected, the primary lesion is generally small in size, difficult to be detected and often located at an anatomic region known as the gastrinoma triangle [20]. In the presence of paraneoplastic syndrome, including ectopic ACTH syndrome, a primary tumor located in the lung, the thyroid (medullary carcinoma) or associated with a gastrinoma should be suspected [21]. However, specific biomarkers for UPO-NENs are still lacking.

In clinical practice, the first sign of a neoplastic process secondary to a UPO-NEN is the detection of liver metastases via conventional radiology (i.e., CT scan). Additional work-up, such as upper and lower GI endoscopy, chest CT and MRI of the abdomen, should be required. Conventional radiology might fail to detect the primary tumor in the pancreas or small bowel when the lesions are small or the tests are performed using a suboptimal protocol [22].

3.3.1. Pathology

In patients with UPOs, immunohistochemical markers are useful for cell-type determination and pathologic diagnosis.

UPO-NENs are most often well-differentiated grade 1 or 2 tumors which commonly originate from the intestinal or pancreatic system (approximately 60–65% of cases) or lungs (approximately 20–25%) [7,23]. Liver metastases dominate in the clinical setting, and these lesions are usually reachable using a core-needle biopsy (CNB), as current guidelines strongly recommend; however, occasionally, focal liver resections might be necessary to obtain sufficient material [24]. An example from our experience is as follows: a 50-year-old man's biopsy with a single liver metastasis in apparently occult primary tumor is shown in Figure 2. The histopathological findings of the specimen revealed a well-differentiated neoplasm with a predominantly nested architecture on routine hematoxylin–eosin stain (A), monotonous small-sized cells with round nuclei, finely stippled chromatin and heavy eosinophilic cytoplasmic granularity diagnostic of an enterochromaffin-cell (EC) tumor; 100% of the neoplastic cells showed a strong cytoplasmatic immunoreactivity for general neuroendocrine markers such as Chromogranin-A (B) and Synaptophisin as well as for small intestine site-specific immunohistochemical markers such as *CDX2* (C) and serotonin (D); tumors with this morphology and immunohistochemical profile typically arise in the ileum (metastatic small-intestine well-differentiated NEN, SI-WDNEN).
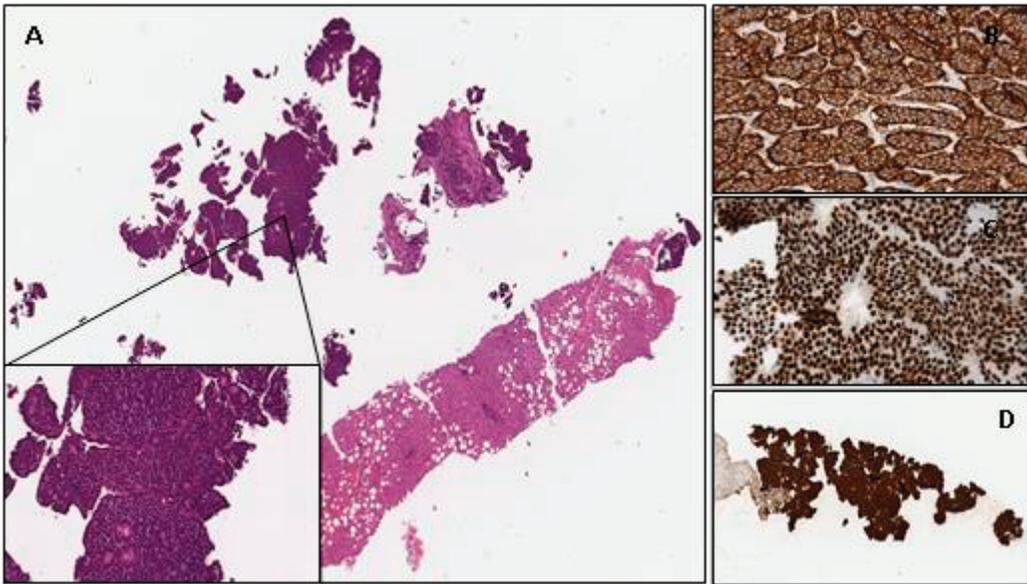
Figure 2. Histological and immunohistochemical attributes of a metastatic small-intestinal NET (SI-NET). (**A**), Characteristic organoid or nested architectural pattern of well-differentiated neuroendocrine tumor. Immunohistochemical expression was noted for Chromogranin (**A**,**B**), CDX2 (**C**) and Serotonin (**D**).

In summary, morphological, immunohistochemical and molecular analyses are equally essential in the assessment of NENs. NENs may exhibit variable growth patterns and cellular characteristics easily identifiable on routine hematoxylin–eosin staining alone [25,26]. For instance, while metastatic NENs with a primary tumor located in the stomach and duodenum may demonstrate a glandular-like pattern, and SI-NENs often exhibit an organoid or nested growth pattern; in contrast, pancreatic and rectal NENs may present with a ribbon-like or trabecular architecture (Figure 3). To identify the actual origin of a UPO-NEN, a wide variety of immunohistochemical markers may be assessed. These include classic markers such as Chromogranin A (CgA) and Synaptophysin (SYP) or INSM1 [27] to confirm the neuroendocrine differentiation [28,29]. CDX2 is a transcription factor, a useful marker of intestinal NENs and, because of its association with GI differentiation, it is also found in gastrin-positive pancreatic NENs and colorectal adenocarcinoma [30,31]. In the setting of WDNENs, Thyroid Transcription Factor1 (TTF1) positivity may suggest a bronchial primary in 43% of the cases. However, it is not specific in poorly differentiated lung neuroendocrine carcinomas (PDNECs), as it is also present in 50% of small-cell tumors at other sites [30,32,33]. Islet-1 (ISL1) can be used as a marker for pancreatic origin [34,35]. Serotonin, associated with CDX2 and SATB2, has utility in identifying EC tumors originating in the ileum or appendix [36,37]. Colorectal NENs may present with positive staining for glucagon-like peptide 1, CDX2 and SATB2 [35,38]. Pheochromocytomas and abdominal paragangliomas stain positive for neuroendocrine markers CgA, SYP, ISL1, INSM1 and, often, GATA3; subsets of cases may display an intricate network of supporting sustentacular cells which are highlighted by an S100 or SOX10 stain [39]. Paranuclear dot-like staining for CgA, CK20 and Neurofilament (NF), and polyomavirus stain, may also help in the identification of neuroendocrine skin lesions such as Merkel cell carcinomas (MCCs) [40,41]. Therefore, to successfully identify UPO-NENs, a combined assessment using clues from clinical history, radiology, morphology and immunohistochemistry is

recommended, rather than blind trust in a single marker [12,42]. The interaction between physicians and pathologists is, therefore, fundamental.
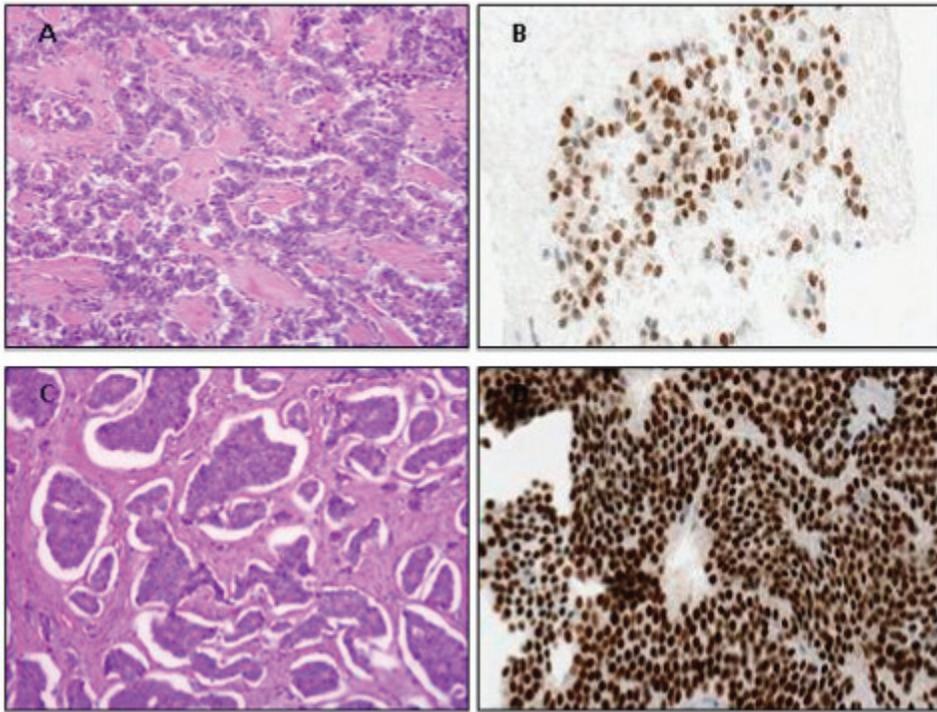

Figure 3. (**A**), Characteristic ribbon-like architectural pattern of pancreatic well-differentiated neuroendocrine tumor with site-specific ISLET-1 immunostaining positivity outline pancreatic landscape (**B**); in contrast, intestinal NENs usually present an organoid morphological pattern (**C**) with immunoreactivity for site-specific marker CDX2 in an intestinal landscape (**D**).

3.3.2. Functional Imaging

Somatostatin receptor scintigraphy (SRS) has been extensively used for the initial staging of disease and to evaluate somatostatin receptor (SSTRs) status; furthermore, it has been explored to detect occult primary sites in patients with metastatic gastro-entero-pancreatic (GEP) NENs with a detection rate of 39%. However, 68GaDOTANOC positron emission tomography (PET)/CT has proved to be more accurate and generally represents the functional imaging of choice, being able to also detect very small lesions [43]. According to previous experiences, Ga-68-DOTANOC PET/CT helped in the detection of undiagnosed primary sites in patients with metastatic NENs in a percentage ranging from 45.5% [44] to 59% of the patients [45,46].

A recent meta-analysis [47], including 10 studies of a total of 484 patients with UPO-NENs, demonstrated the high diagnostic sensitivity of 68Ga-DOTA-SSTR for UPO-NENs. 68Ga-DOTA-SSTR PET/CT was highly effective in locating the primary and metastatic sites of UPO-NENs, with a pooled detection rate of 61%.

Fluorodeoxyglucose PET may be employed for the detection of occult primary sites in case of high-grade histology (G3 NEN), whereas F-DOPA and MIBG imaging may be employed in selected cases, especially when paraganglioma/pheochromocytoma are suspected.

3.3.3. Capsule Endoscopy (CE) and Double-Balloon Enteroscopy (DBE)

SI-NENs have always been considered difficult to diagnose in view of their non-specific presentation and poor accessibility of the distal small bowel [48].

Conventional radiology (with or without enteroclysis) is often not accurate enough in the detection of SI-NENs [45], whilst PET/CT with 68Ga-DOTA peptides, despite being the most accurate modality in the detection of well-differentiated NENs, does not allow to obtain a histological diagnosis and might be unable to differentiate between intestinal and mesenteric localization [49].

Capsule endoscopy (CE) and double-balloon enteroscopy (DBE) have significantly improved the diagnosis of SI-NENs, although their use is still limited in routine clinical practice and data on their actual safety and efficacy in the neuroendocrine setting are scant [50]. In a retrospective study [51], in 11 patients with UPO-NEN, CE identified lesions suggestive of small-bowel primary in 8/10 patients in whom it was successful, and all these tumors were histologically confirmed. Conversely, in a recent prospective study by Furnari et al. [52], in 24 patients with a histological diagnosis of metastatic NEN of UPO, the diagnostic yield of CE was compared with the surgical exploration. CE identified a primary SI-NEN in eleven subjects, but the final diagnosis of SI-NEN was confirmed only in five cases after surgical exploration. It is likely that the high number of false-positive results might have been the consequence of confounding factors, including small-bowel contractions, extrinsic compression and lymph stasis.

DBE is more invasive when compared with CE, but allows to determine the precise location as well as the actual number of tumors, and, more importantly, to obtain biopsies for obtaining a pathological diagnosis. In a study involving 12 patients with suspected SI-NEN or with liver NEN metastases, who underwent DBE, a diagnostic yield of DBE for primary tumor of 33% was reported [53]. In five patients with metastatic midgut NENs who underwent DBE, a NEN of the ileum was detected and histologically confirmed in four out of the five patients, whereas conventional radiological imaging did not visualize any of the primary tumors [54]. In a recent prospective study [55], sensitivity and specificity for DBE in detecting SI-NEN were reported to be of 60% and 100%, respectively. According to the available data, DBE should be the technique of choice in the pre-surgical setting given the high specificity, also considering that it allows one to obtain a histological diagnosis. However, further studies are warranted to clarify the actual role of CE and DBE in the diagnostic algorithm of UPO-NENs.

3.3.4. Ultrasound Endoscopy (EUS)

Ultrasound endoscopy (EUS) represents the diagnostic gold standard for pancreatic NENs with an up-to-94% sensitivity [56] and is the technique of choice for the locoregional staging of gastric, duodenal and rectal NENs. Notably, EUS sensitivity in detecting pancreatic NENs is higher than the CT scan or MRI pancreatic lesion detection rate [57]. According to a recent systematic review and meta-analysis, the adjusted incremental benefit of pre-operative EUS for the detection of suspected pancreatic NENs after other investigative modalities had failed was 26% and EUS allowed for the identification of pancreatic NENs in 97% of the cases [58].

A possible diagnostic algorithm for the detection of NENs of UPO is proposed in Figure 4.
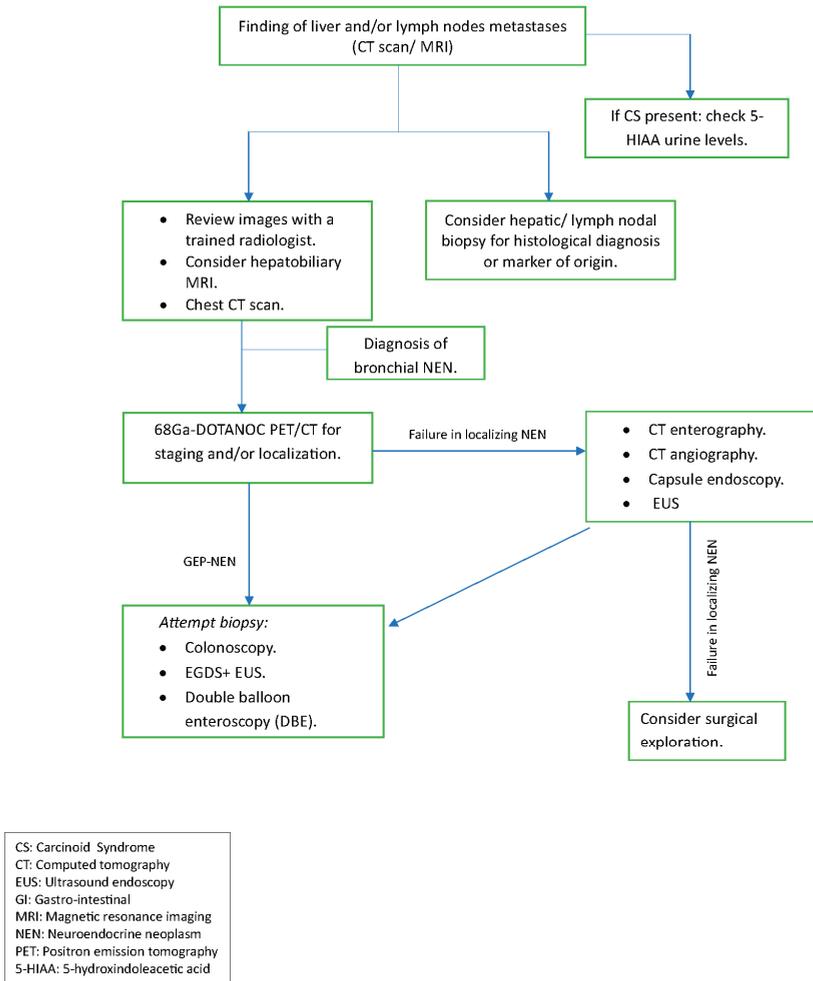
Figure 4. A possible diagnostic algorithm for the detection of neuroendocrine neoplasms of unknown origin.

4. Discussion

UPO-NENs constitute 11% to 14% of all GEP-NENs, representing a challenging entity for both diagnosis and treatment. Early-identification of the primary tumor is necessary to define a patient's management and prognosis [4], taking into account that the resection of the primary tumor even in a metastatic disease is generally correlated with a better survival [6]. This is particularly true for SI-NEN, as the resection of the primary tumor also reduces the risk of complications (i.e., intestinal sub-occlusion/occlusion, abdominal pain due to mesenteric fibrosis), taking into account that this kind of surgery is characterized by low morbidity and mortality. On the other hand, there are fewer clear-cut indications whenever the primary tumor is located in the pancreas and the patient is metastatic, considering the major complications that may follow pancreatic surgery.

The majority of UPO-NENs are found in the small bowel [9,10]. However, localization of midgut tumors might be challenging due to their usually small size and the impaired accessibility of the small bowel to standard endoscopic techniques [48]. The clinical presentation is often non-specific and not particularly useful for diagnosis; however, in the

case of functioning tumors, the presence of CS, also diagnosed through elevated levels of 5HIAA, or the presence of symptoms typical of pancreatic/duodenal functioning NENs (see Table 1) might be of help in identifying the site of the primary tumor. In the diagnostic and staging phase, 68GaPET is fundamental. This is particularly true for asymptomatic UPO-NENs and/or whenever pathology is not helpful, also considering its high diagnostic accuracy in the detection of very small lesions [43] and the ability to detect undiagnosed primary sites in patients with metastatic NENs in up to 59% of cases [45,46]. In this scenario, a multimodal approach including conventional radiology and more sophisticated techniques such as CT enterography and CT angiography, nuclear medicine and endoscopy is necessary.

In the specific setting of UPO-NENs, the role of pathology is essential to provide useful information which can orientate clinicians in the search for the primary site. In this context, a wide variety of immunohistochemical markers may be assessed, as previously described. From a practical point of view, if CDX2 is expressed, an intestinal origin is more likely [30], suggesting the need for endoscopic procedures including VCE and DBE in order to find the primary tumor, the latter also allowing to achieve histological confirmation. Whenever Islet-1 (ISL1) is expressed, a pancreatic origin is suggested [34] and, in this case, EUS should be the diagnostic tool of choice, being the gold standard for pancreatic NENs with an up-to-94% sensitivity [56]. Finally, a lymph node or liver metastasis with Thyroid Transcription Factor1 (TTF1) positivity may suggest a bronchial primary in 43% of the cases; however, the role of immunohistochemical markers could be less accurate in poorly differentiated tumors [30,32]. It is, indeed, clear that the proper interaction between clinicians and pathologists is fundamental for the management of these tumors.

As a future perspective, molecular biology and gene-expression profiling represent a promising and growing area in order to obtain a subtype-specific NEN molecular-landscape characterization, that, together with clinical and pathological data, may help to determine tumor origin in UPO-NENs and, even more importantly, might allow one to identify molecular treatment targets.

5. Conclusions

In summary, the management of UPO-NENs is challenging, taking into account that therapeutic options are limited. Multidisciplinary management in the diagnostic setting, including a strict cooperation between clinicians and pathologists, plays a key role in this setting, even more than in other NENs. The identification of the primary tumor is warranted, particularly in well-differentiated forms, in order to improve survival and allow access to adequate treatment options. As a matter of fact, once the primary tumor has been removed, generally limiting the disease to the liver, viable curative strategies are available, including liver resection and, in highly selected cases, liver transplantation. Furthermore, some treatment options including targeted therapies and radiopeptide treatment may be limited by registrative boundaries in UPO-NENs. Further studies, possibly prospective and randomized, are needed to improve the management of these tumors, with a specific focus on molecular biology and gene-expression profiling, which might be of great help for both the diagnosis and the treatment of these neoplasms.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

References

1. Bhosale, P.; Shah, A.; Wei, W.; Varadhachary, G.; Johnson, V.; Shah, V.; Kundra, V. Carcinoid tumours: Predicting the location of the primary neoplasm based on the sites of metastases. *Eur. Radiol.* **2013**, *23*, 400–407. [CrossRef] [PubMed]
2. Yao, J.C.; Hassan, M.; Phan, A.; Dagohoy, C.; Leary, C.; Abdalla, J.E.M.K.; Fleming, J.B.; Vauthey, J.-N.; Rashid, A.; Evans, D.B. One hundred years after "carcinoid": Epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J. Clin. Oncol.* **2008**, *26*, 3063–3072. [CrossRef] [PubMed]
3. Stella, G.M.; Senetta, R.; Cassenti, A.; Ronco, M.; Cassoni, P. Cancers of unknown primary origin: Current perspectives and future therapeutic strategies. *J. Transl. Med.* **2012**, *10*, 12. [CrossRef]
4. Stoyianni, A.; Pentheroudakis, G.; Pavlidis, N. Neuroendocrine carcinoma of unknown primary: A systematic review of the literature and a comparative study with other neuroendocrine tumors. *Cancer Treat. Rev.* **2011**, *37*, 358–365. [CrossRef] [PubMed]
5. Ahmed, A.; Turner, G.; King, B.; Jones, L.; Culliford, D.; McCance, D.; Ardill, J.; Johnston, B.T.; Poston, G.; Rees, M.; et al. Midgut neuroendocrine tumours with liver metastases: Results of the UKINETS study. *Endocr. Relat. Cancer* **2009**, *16*, 885–894. [CrossRef] [PubMed]
6. Citterio, D.; Pusceddu, S.; Facciorusso, A.; Coppa, J.; Milione, M.; Buzzoni, R.; Bongini, M.; Debraud, F.; Mazzaferro, V. Primary tumour resection may improve survival in functional well-differentiated neuroendocrine tumours metastatic to the liver. *Eur. J. Surg. Oncol.* **2017**, *43*, 380–387. [CrossRef] [PubMed]
7. Taal, B.G.; Visser, O. Epidemiology of Neuroendocrine Tumours. *Neuroendocrinology* **2004**, *80*, 3–7. [CrossRef]
8. Rindi, G.; Bordi, C.; Rappel, S.; La Rosa, S.; Stolte, M.; Solcia, E. Gastric carcinoids and neuroendocrine carcinomas: Path-ogenesis, pathology, and behavior. *World J. Surg.* **1996**, *20*, 168–172. [CrossRef]
9. Pavel, M.; O'Toole, D.; Costa, F.; Capdevila, J.; Gross, D.; Kianmanesh, R.; Krenning, E.; Knigge, U.; Salazar, R.; Pape, U.-F.; et al. ENETS Consensus Guidelines Update for the Management of Distant Metastatic Disease of Intestinal, Pancreatic, Bronchial Neuroendocrine Neoplasms (NEN) and NEN of Unknown Primary Site. *Neuroendocrinology* **2016**, *103*, 172–185. [CrossRef]
10. Dasari, A.; Shen, C.; Halperin, D.; Zhao, B.; Zhou, S.; Xu, Y.; Shih, T.; Yao, J.C. Trends in the Incidence, Prevalence, and Survival Outcomes in Patients with Neuroendocrine Tumors in the United States. *JAMA Oncol.* **2017**, *3*, 1335. [CrossRef]
11. Abdel-Rahman, O. A Real-World, Population-Based Study for the Incidence and Outcomes of Neuroendocrine Neoplasms of Unknown Primary. *Neuroendocrinology* **2021**, *111*, 876–882. [CrossRef] [PubMed]
12. Berner, A.M. Nostic Approaches to Neuroendocrine Neoplasms of Unknown Primary Site. *Neuroendocrinology* **2020**, *110*, 563–573. [CrossRef] [PubMed]
13. Datta, S.; Williams, N.; Suortamo, S.; Mahmood, A.; Oliver, C.; Hedley, N.; Ray, P. Carcinoid syndrome from small bowel endocrine carcinoma in the absence of hepatic metastasis. *Age Ageing* **2011**, *40*, 760–762. [CrossRef] [PubMed]
14. Halperin, D.M.; Shen, C.; Dasari, A.; Xu, Y.; Chu, Y.; Zhou, S.; Shih, Y.-C.T.; Yao, J.C. Frequency of carcinoid syndrome at neuroendocrine tumour diagnosis: A population-based study. *Lancet Oncol.* **2017**, *18*, 525–534. [CrossRef] [PubMed]
15. Ramage, J.K.; Ahmed, A.; Ardill, J.; Bax, N.; Breen, D.J.; Caplin, M.E.; Corrie, P.; Davar, J.; Davies, A.H.; Lewington, V.; et al. Guidelines for the management of gastroenteropancreatic neuroendocrine (including carcinoid) tumours (NETs). *Gut* **2012**, *61*, 6–32. [CrossRef]
16. Alexandraki, K.; Angelousi, A.; Boutzios, G.; Kyriakopoulos, G.; Rontogianni, D.; Kaltsas, G. Management of neuroendocrine tumors of unknown primary. *Rev. Endocr. Metab. Disord.* **2017**, *18*, 423–431. [CrossRef]
17. Wang, S.C.; Parekh, J.R.; Zuraek, M.B.; Venook, A.P.; Bergsland, E.K.; Warren, R.S.; Nakakura, E.K. Identification of Unknown Primary Tumors in Patients with Neuroendocrine Liver Metastases. *Arch. Surg.* **2010**, *145*, 276–280. [CrossRef]
18. Massimino, K.P.; Han, E.; Pommier, S.J.; Pommier, R.F. Laparoscopic surgical exploration is an effective strategy for locating occult primary neuroendocrine tumors. *Am. J. Surg.* **2012**, *203*, 628–631. [CrossRef]
19. Spada, F.; Rossi, R.E.; Kara, E.; Laffi, A.; Massironi, S.; Rubino, M.; Grimaldi, F.; Bhoori, S.; Fazio, N. Carcinoid Syndrome and Hy-perinsulinemic Hypoglycemia Associated with Neuroendocrine Neoplasms: A Critical Review on Clinical and Pharmacological Management. *Pharmaceuticals* **2021**, *14*, 539. [CrossRef]
20. Rossi, R.E.; Elvevi, A.; Citterio, D.; Coppa, J.; Invernizzi, P.; Mazzaferro, V.; Massironi, S. Gastrinoma and Zollinger Ellison syndrome: A roadmap for the management between new and old therapies. *World J. Gastroenterol.* **2021**, *27*, 5890–5907. [CrossRef]
21. Wu, Y.; Xiong, G.; Zhang, H.; Wang, M.; Zhu, F.; Qin, R. Adrenocorticotropic Hormone-Producing Pancreatic Neuroendocrine Neoplasms: A Systematic Review. *Endocr. Pract.* **2021**, *27*, 152–157. [CrossRef] [PubMed]
22. Bergsland, E.K.; Nakakura, E.K. Neuroendocrine Tumors of Unknown Primary: Is the Primary Site Really Not Known? *JAMA Surg.* **2014**, *149*, 889. [CrossRef] [PubMed]
23. Catena, L.; Bichisao, E.; Milione, M.; Valente, M.; Platania, M.; Pusceddu, S.; Ducceschi, M.; Zilembo, N.; Formisano, B.; Bajetta, E. Neuroendocrine tumors of unknown primary site: Gold dust or misdiagnosed neoplasms? *Tumori J.* **2011**, *97*, 564–567. [CrossRef] [PubMed]
24. Pavel, M.; Baudin, E.; Couvelard, A.; Krenning, E.; Öberg, K.; Steinmüller, T.; Anlauf, M.; Wiedenmann, B.; Salazar, R. ENETS Consensus Guidelines for the Management of Patients with Liver and Other Distant Metastases from Neuroendocrine Neoplasms of Foregut, Midgut, Hindgut, and Unknown Primary. *Neuroendocrinology* **2012**, *95*, 157–176. [CrossRef] [PubMed]
25. WHO Classification of Tumours Editorial Board. *WHO Classification of Tumours. Digestive System Tumours*, 5th ed.; WHO Classification of Tumours Editorial Board: Geneva, Switzerland, 2021.

26. WHO Classification of Tumours of Endocrine Organs. *World Health Organization Classification of Tumours*, 4th ed.; WHO Classification of Tumours Editorial Board: Geneva, Switzerland, 2017.

27. Lilo, M.T.; Chen, Y.; LeBlanc, R.E. INSM1 Is More Sensitive and Interpretable than Conventional Immunohistochemical Stains Used to Diagnose Merkel Cell Carcinoma. *Am. J. Surg. Pathol.* **2018**, *42*, 1541–1548. [CrossRef]

28. Oien, K.A. Pathologic Evaluation of Unknown Primary Cancer. *Semin. Oncol.* **2009**, *36*, 8–37. [CrossRef]

29. Kriegsmann, K.; Zgorzelski, C.; Muley, T.; Christopoulos, P.; Thomas, M.; Winter, H.; Eichhorn, M.; Eichhorn, F.; von Winterfeld, M.; Herpel, E.; et al. Role of Synaptophysin, Chromogranin and CD56 in adenocarcinoma and squamous cell carcinoma of the lung lacking morphological features of neuroendocrine differentiation: A retrospective large-scale study on 1170 tissue samples. *BMC Cancer* **2021**, *21*, 486. [CrossRef]

30. Lin, X.; Saad, R.S.; Luckasevic, T.M.; Silverman, J.F.; Liu, Y. Diagnostic Value of CDX-2 and TTF-1 Expressions in Separating Metastatic Neuroendocrine Neoplasms of Unknown Origin. *Appl. Immunohistochem. Mol. Morphol.* **2007**, *15*, 407–414. [CrossRef]

31. Silberg, D.G.; Swain, G.P.; Suh, E.R.; Traber, P.G. Cdx1 and Cdx2 expression during intestinal development. *Gastroenterology* **2000**, *119*, 961–971. [CrossRef]

32. Jagirdar, J. Application of Immunohistochemistry to the Diagnosis of Primary and Metastatic Carcinoma to the Lung. *Arch. Pathol. Lab. Med.* **2008**, *132*, 384–396. [CrossRef]

33. Lazzaro, D.; Price, M.; Felice, M.D.; Lauro, R.D. The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **1991**, *113*, 1093–1104. [CrossRef] [PubMed]

34. Koo, J.; Mertens, R.B.; Mirocha, J.M.; Wang, H.L.; Dhall, D. Value of Islet 1 and PAX8 in identifying metastatic neuroendocrine tumors of pancreatic origin. *Mod. Pathol.* **2012**, *25*, 893–901. [CrossRef] [PubMed]

35. Koo, J.; Zhou, X.; Moschiano, E.; De Peralta-Venturina, M.; Mertens, R.B.; Dhall, D. The Immunohistochemical Expression of Islet 1 and PAX8 by Rectal Neuroendocrine Tumors Should Be Taken into Account in the Differential Diagnosis of Metastatic Neuroendocrine Tumors of Unknown Primary Origin. *Endocr. Pathol.* **2013**, *24*, 184–190. [CrossRef] [PubMed]

36. Solcia, E.; Vanoli, A. Histogenesis and Natural History of Gut Neuroendocrine Tumors: Present Status. *Endocr. Pathol.* **2014**, *25*, 165–170. [CrossRef]

37. Bellizzi, A.M. SATB2 in neuroendocrine neoplasms: Strong expression is restricted to well-differentiated tumours of lower gastrointestinal tract origin and is most frequent in Merkel cell carcinoma among poorly differentiated carcinomas. *Histopathology* **2020**, *76*, 251–264. [CrossRef] [PubMed]

38. Kim, J.Y.; Kim, K.-S.; Kim, K.-J.; Park, I.J.; Lee, J.L.; Myung, S.-J.; Park, Y.; Park, Y.S.; Yu, C.S.; Kim, J.C.; et al. Non-L-cell Immunophenotype and Large Tumor Size in Rectal Neuroendocrine Tumors Are Associated With Aggressive Clinical Behavior and Worse Prognosis. *Am. J. Surg. Pathol.* **2015**, *39*, 632–643. [CrossRef]

39. Juhlin, C.C. Challenges in Paragangliomas and Pheochromocytomas: From Histology to Molecular Immunohistochemistry. *Endocr. Pathol.* **2021**, *32*, 228–244. [CrossRef]

40. Shuda, M.; Arora, R.; Kwun, H.J.; Feng, H.; Sarid, R.; Fernández-Figueras, M.-T.; Tolstov, Y.; Gjoerup, O.; Mansukhani, M.M.; Swerdlow, S.H.; et al. Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors. *Int. J. Cancer* **2009**, *125*, 1243–1249. [CrossRef]

41. Kuhajda, F.P.; Olson, J.L.; Mann, R.B. Merkel cell (small cell) carcinoma of the skin: Immunohistochemical and ultrastructural demonstration of distinctive perinuclear cytokeratin aggregates and a possible association with B cell neoplasms. *Histochem. J.* **1986**, *18*, 239–244. [CrossRef]

42. Juhlin, C.C.; Zedenius, J.; Höög, A. Metastatic Neuroendocrine Neoplasms of Unknown Primary: Clues from Pathology Workup. *Cancers* **2022**, *14*, 2210. [CrossRef]

43. Pellegrino, F.; Granata, V.; Fusco, R.; Grassi, F.; Tafuto, S.; Perrucci, L.; Tralli, G.; Scaglione, M. Diagnostic Management of Gastroenteropancreatic Neuroendocrine Neoplasms: Technique Optimization and Tips and Tricks for Radiologists. *Tomography* **2023**, *9*, 217–246. [CrossRef]

44. Schreiter, N.F.; Bartels, A.-M.; Froeling, V.; Steffen, I.; Pape, U.-F.; Beck, A.; Hamm, B.; Brenner, W.; Röttgen, R. Searching for primaries in patients with neuroendocrine tumors (NET) of unknown primary and clinically suspected NET: Evaluation of Ga-68 DOTATOC PET/CT and In-111 DTPA octreotide SPECT/CT. *Radiol. Oncol.* **2014**, *48*, 339–347. [CrossRef]

45. Prasad, V.; Ambrosini, V.; Hommann, M.; Hoersch, D.; Fanti, S.; Baum, R.P. Detection of unknown primary neuroendocrine tumours (CUP-NET) using 68Ga-DOTA-NOC receptor PET/CT. *Eur. J. Nucl. Med. Mol. Imaging* **2010**, *37*, 67–77. [CrossRef]

46. Pruthi, A.; Pankaj, P.; Verma, R.; Jain, A.; Belho, E.S.; Mahajan, H. Ga-68 DOTANOC PET/CT imaging in detection of primary site in patients with metastatic neuroendocrine tumours of unknown origin and its impact on clinical decision making: Experience from a tertiary care centre in India. *J. Gastrointest. Oncol.* **2016**, *7*, 449–461. [CrossRef] [PubMed]

47. Ma, H.; Kan, Y.; Yang, J. Clinical value of ^{68}Ga-DOTA-SSTR PET/CT in the diagnosis and detection of neuroendocrine tumors of unknown primary origin: A systematic review and meta-analysis. *Acta Radiol.* **2021**, *62*, 1217–1228. [CrossRef] [PubMed]

48. Rossi, R.E.; Conte, D.; Elli, L.; Branchi, F.; Massironi, S. Endoscopic techniques to detect small-bowel neuroendocrine tumors: A literature review. *United Eur. Gastroenterol. J.* **2017**, *5*, 5–12. [CrossRef] [PubMed]

49. Sharma, P.; Arora, S.; Mukherjee, A.; Pal, S.; Sahni, P.; Garg, P.; Khadgawat, R.; Thulkar, S.; Bal, C.; Kumar, R. Predictive Value of 68Ga-DOTANOC PET/CT in Patients with Suspicion of Neuroendocrine Tumors: Is Its Routine Use Justified? *Clin. Nucl. Med.* **2014**, *39*, 37–43. [CrossRef] [PubMed]

50. Rossi, R.E.; Elvevi, A.; Gallo, C.; Palermo, A.; Invernizzi, P.; Massironi, S. Endoscopic techniques for diagnosis and treatment of gastro-entero-pancreatic neuroendocrine neoplasms: Where we are. *World J. Gastroenterol.* **2022**, *28*, 3258–3273. [CrossRef]
51. Frilling, A.; Smith, G.; Clift, A.K.; Martin, J. Capsule endoscopy to detect primary tumour site in metastatic neuroendocrine tumours. *Dig. Liver Dis.* **2014**, *46*, 1038–1042. [CrossRef]
52. Furnari, M.; Buda, A.; Delconte, G.; Citterio, D.; Voiosu, T.; Ballardini, G.; Cavallaro, F.; Savarino, E.; Mazzaferro, V.; Meroni, E. The Role of Wireless Capsule Endoscopy (WCE) in the Detection of Occult Primary Neuroendocrine Tumors. *J. Gastrointestin. Liver Dis.* **2017**, *26*, 151–156. [CrossRef]
53. Bellutti, M.; Fry, L.C.; Schmitt, J.; Seemann, M.; Klose, S.; Malfertheiner, P.; Mönkemüller, K. Detection of Neuroendocrine Tumors of the Small Bowel by Double Balloon Enteroscopy. *Dig. Dis. Sci.* **2009**, *54*, 1050–1058. [CrossRef]
54. Scherübl, H.; Faiss, S.; Tschöpe, R.; Zeitz, M. Double-balloon enteroscopy for the detection of midgut carcinoids. *Gastrointest. Endosc.* **2005**, *62*, 994. [CrossRef] [PubMed]
55. Rossi, R.E.; Elli, L.; Branchi, F.; Conte, D.; Massironi, S. Double-Balloon Enteroscopy in Detecting Small-Bowel Neuroendocrine Neoplasms: A Single-Center Prospective Study. *Digestion* **2021**, *102*, 722–730. [CrossRef] [PubMed]
56. Falconi, M.; Eriksson, B.; Kaltsas, G.; Bartsch, D.K.; Capdevila, J.; Caplin, M.; Kos-Kudla, B.; Kwekkeboom, D.; Rindi, G.; Klöppel, G.; et al. ENETS Consensus Guidelines Update for the Management of Patients with Functional Pancreatic Neuroendocrine Tumors and Non-Functional Pancreatic Neuroendocrine Tumors. *Neuroendocrinology* **2016**, *103*, 153–171. [CrossRef] [PubMed]
57. Manta, R.; Nardi, E.; Pagano, N.; Ricci, C.; Sica, M.; Castellani, D.; Bertani, H.; Piccoli, M.; Mullineris, B.; Tringali, A.; et al. Pre-operative Diagnosis of Pancreatic Neuroendocrine Tumors with Endoscopic Ultrasonography and Computed Tomography in a Large Series. *J. Gastrointestin. Liver Dis.* **2016**, *25*, 317–321. [CrossRef]
58. James, P.D.; Tsolakis, A.V.; Zhang, M.; Belletrutti, P.J.; Mohamed, R.; Roberts, D.J.; Heitman, S.J. Incremental benefit of preoperative EUS for the detection of pancreatic neuroendocrine tumors: A meta-analysis. *Gastrointest. Endosc.* **2015**, *81*, 848–856.e1. [CrossRef]

Case Report

Pancreatic Neuroendocrine Tumor (Pan-NET) Presented by Abdominal Pain: A Case Report and Literature Review

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Abstract: A pancreatic neuroendocrine tumor (Pan-NET) is a rare neoplasm originating in the neuroendocrine system. Carcinoid syndrome occurs in approximately 19% of patients with functional Pan-NETs, typically when liver metastases occur. In this paper, we describe the case of a patient with a low-grade non-functional Pan-NET, but with a typical clinical presentation of carcinoid syndrome. An 81-year-old male was admitted to our Department of Internal Medicine at Cannizzaro Hospital (Catania, Italy) because of the onset of abdominal pain with nausea, loose stools, and episodic flushing. Firstly, an abdominal contrast-enhanced CT scan showed a small pancreatic hypervascular mass; then, a gallium-68 DOTATOC integrated PET/CT revealed an elevated expression of SSTR receptors. Serum chromogranin A and urinary 5-HIAA measurements were negative. We performed an endoscopic ultrasonography (EUS) by a fine-needle biopsy (EUS-FNB), allowing the immunostaining of a small mass (0.8 cm) and the diagnosis of a low-grade (G1) non-functional Pan-NET (NF-Pan-NET). Surgery was waived, while a follow-up strategy was chosen. The early recognition of Pan-NETs, although rare, is necessary to improve the patient's survival. Although helpful to allow for immunostaining, EUS-FNB needs to be warranted in future studies comparing EUS-FNB to EUS-FNA (fine-needle aspiration), which is, to date, reported as the tool of choice to diagnose Pan-NETs.

Keywords: neuroendocrine tumors (NETs); pancreatic neuroendocrine tumors (Pan-NETs); endoscopic ultrasonography; EUS-FNA; EUS-FNB

1. Introduction

Neuroendocrine neoplasms (NENs) are enigmatic malignancies with an increasing incidence and prevalence [1–4]. Given their common morphological and immunophenotypical features, all these tumors arise from cells of the diffuse endocrine system.

NENs range from asymptomatic well-differentiated neuroendocrine tumors (NETs) to aggressive neuroendocrine carcinomas (NECs). In fact, nearly 80–90% of NENs are NETs, while the remaining 10–20% are carcinomas [5].

NETs can develop in any tissue of the body. The gastrointestinal tract and pancreas are the most common sites of origin, accounting for approximately 60% of the primary sites [6], followed by the lungs and other sites.

About 40% of NETs can release hormones responsible for symptoms, depending on the secreted hormone. Carcinoid syndrome is characterized by episodic flushing and diarrhea,

due to various vasoactive substances (serotonin, histamine, and other amines) released into the systemic circulation [7].

Non-functional NETs may often present with subtle and sporadic symptoms, sometimes with gastrointestinal bleeding, abdominal pain, bowel obstruction, or unexplained weight loss [8].

Treatment and prognosis depend on the grade and stage of the tumor. NETs diagnosis is frequently late, along with symptoms related to hormone hypersecretion, often after metastases occurs in the liver, where bioactive substances fail to be inactivated. An early diagnosis and recognition are necessary to improve the patient's survival, which has not significantly changed over the last 30 years [9].

In this paper, we present a case of a pauci-symptomatic pancreatic neuroendocrine tumor in a patient with an unspecific clinical presentation (abdominal pain) and mild additional symptoms (nausea and loose stools). This was the occasion for a narrative review of the literature on the diagnosis and management of pancreatic neuroendocrine tumors (Pan-NETs).

2. Case-Report

In May 2023, an 81-year-old man was admitted to the Department of Internal Medicine at Cannizzaro Hospital (Catania, Italy) because of the onset of abdominal pain, especially in the lower abdominal quadrants, with nausea and loose stools (<3 times/day).

The patient's past medical history included arterial hypertension, type-2 diabetes mellitus, peripheral artery disease (PAD), obesity, hypothyroidism, and depressive syndrome. In the past six months, he complained of abdominal distension and changes in bowel habits (loose stools). There was no relevant family history. He was taking levothyroxine, insulin according to HGT, lansoprazole, acarbose, ezetimibe/simvastatin, and furosemide. He denied the anamnestic consumption of uncooked meat, fish, or unpasteurized dairy products.

On admission, he had no fever, arterial hypertension (177/76 mmHg), had a normal heart rate (86 bpm), glycemia of 102 mg/dL, and normal SaO_2 in room air (98%); he presented no sensorium alterations. A physical examination revealed abdominal distension, with colic pain on deep palpation and hypoactive abdomen sounds. Mucous membranes were normally hydrated. The bedside FAST (Focused Assessment with Sonography in Trauma) scan did not detect peritoneal fluid. The digital rectal examination showed blood traces.

Laboratory tests were performed, showing an increase in serum CRP (17.9 mg/dL), moderate leukocytosis, moderate renal dysfunction (serum Cr: 1.33 mg/dL, eGFR: 54 mL/min/ $1.73 \, m^2$), normal serum potassium (3.6 mEq/L), sodium (139 mEq/L) and chloride (100 mEq/L), mild metabolic acidosis (pH: 7.33, HCO_3: 21 mmol/L, pCO_2: 42 mmHg), and serum procalcitonin < 0.2 ng/mL. Infectious causes of diarrhea were excluded by microbiological and chemical fecal examinations. An abdomen X-ray excluded bowel obstruction or perforation. Moderate intravenous fluid repletion was administered.

A few hours after admission, the patient experienced transient states of agitation, with uncontrolled crying spells and temper tantrums. Due to his past medical history of untreated depression, anxiolytic and antipsychotic therapies were prescribed, followed by poor efficacy. During this altered emotional status, a flushing episode was observed in the face and neck.

A contrast-enhanced abdominal CT scan revealed a pancreatic hypervascular small mass (8 mm) (Figure 1).

Figure 1. Contrast-enhanced abdominal CT scan: axial section showing a homogeneous and hypervascular mass of 8 mm (red arrow) on the arterial phase.

On the fifth day of admission, given the suspicion of a pancreatic neuroendocrine tumor (Pan-NET), a gallium-68 DOTATOC integrated PET/CT was performed (Figure 2), confirming a small mass between the head and body of the pancreas, with an elevated expression of SSTR2/5 somatostatin receptors. No other sites of disease were detected.
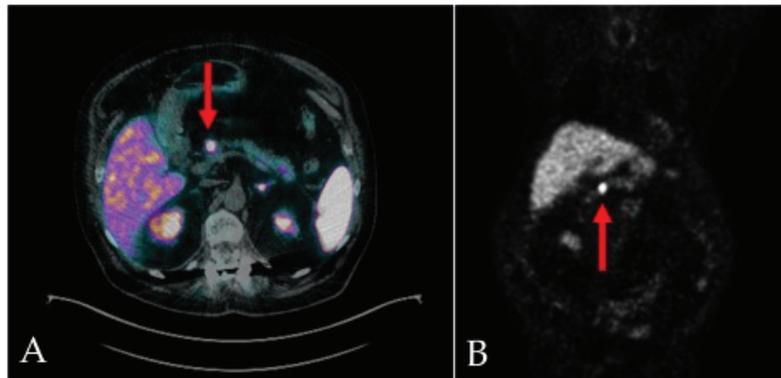

Figure 2. ^{68}Ga-DOTA-TOC integrated PET/CT scans, transaxial (**A**) and MIP (**B**), show focal and intense uptake in the primary pancreatic lesion (red arrows), with an elevated expression of SSTR2/5 somatostatin receptors.

The serum chromogranin A (CgA) measurement was within the normal range (98.0 ng/mL, normal values < 101.9 ng/mL); we also performed a urine 5-HIAA test (urinary 5-HIAA: 1.6 mg/24 h; normal values: 1.0–8.2 mg/24 h).

A progressive recovery was observed, with no further abdominal pain. In accordance with the remission of symptoms and the normal laboratory values, the patient was discharged with the prescription to undergo an endoscopic ultrasonography with a fine-needle biopsy (EUS-FNB) for targeted diagnostic and therapeutic management.

In June 2023, an EUS-FNB, performed with a 22-gauge Acquire needle (Boston Scientific, Marlborough, MA, USA) using a slow-pull technique, visualized the presence of an oval hypo-echogenic mass, with a major axis of 8.9 mm (Figure 3), which was sampled for the cyto-histological examination.
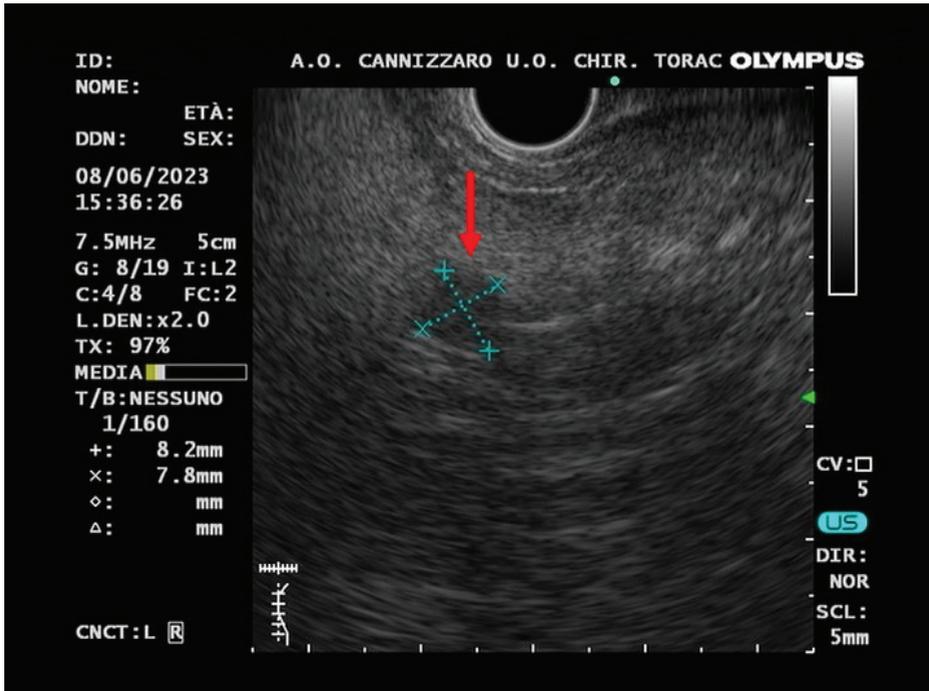

Figure 3. Endoscopic ultrasound (EUS) image (red arrow) of a small hypo-echogenic lesion with a regular margin and a major axis of 8.9 mm.

Histological and immunohistochemical examinations confirmed the suspicion of Pan-NET (stage WHO G1, well-differentiated, synaptophysin positive, CgA positive, Ki67 1%) (Figure 4). The fine-needle biopsy allowed us to obtain microcores of the sample tissue (Figure 4A). Then, using a pipette, the microcores were picked up to be treated as a traditional biopsy. The microcores were composed of abundant blood and entrapped epithelial elements of pancreatic tissue (Figure 4B). A monomorphic population of epithelial cells, in solid sheets or small nodules, with a granular cytoplasm and nuclei with thickened chromatin was also observed (Figure 4C). Immunochemistry, performed with a Bond-Leica immunostainer, revealed positivity for neuroendocrine markers, such as chromogranin A (Figure 4D) and synaptophysin (Figure 4E), while that of serotonin was negative (Figure 4F). The absence of mitosis and necrosis, together with a low Ki-67 index (Figure 4G), allowed us to determine a low-grade neuroendocrine neoplasm.

In keeping with the current guidelines, these findings suggest the diagnosis of a low-grade (G1) non-functional pancreatic neuroendocrine tumor (NF-Pan-NET) (well-differentiated neoplasm, absence of mythosis, Ki67 $\leq$ 2%) [10]. This definition of "non-functional", based only on negative hormone tests, was finalized to a categorical distinction between "secreting" and "non-secreting" tumors, although it underestimated the importance of the clinical presentation.
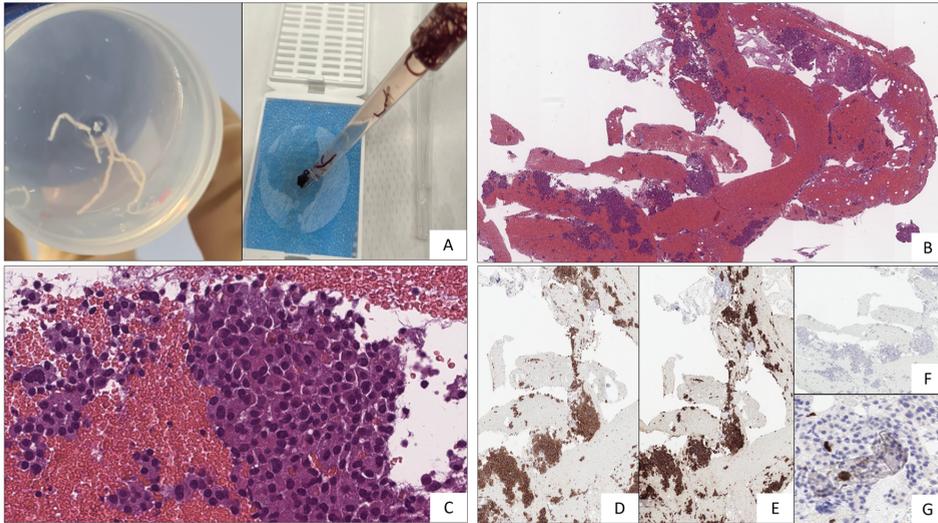
Figure 4. (**A**) Microcores of sample tissue. (**B**) Abundant blood and entrapped epithelial elements of pancreatic tissue stained with Hematoxylin–Eosin. (**C**) Epithelial cells, with a granular cytoplasm and nuclei with thickened chromatin (Hematoxylin–Eosin staining). (**D**) Chromogranin A (5H7 clone, immunohistochemical staining). (**E**) Synaptophysin (27G12 clone, immunohistochemical staining). (**F**) Serotonin (YC5/45 clone, immunohistochemical staining). (**G**) Ki67 (MM1 clone, immunohistochemical staining).

After the evaluations of the stage, grading, symptoms, and comorbidities, a conservative approach of watchful waiting was chosen by the surgeon, with a radiological follow-up session after one year. We scheduled a clinical follow-up session in order to keep the symptoms under observation.

3. Review of the Literature

Neuroendocrine neoplasms (NENs) are heterogenous neoplasms arising in the secretory cells of the diffuse neuroendocrine system, the so-called APUD (Amine Precursor Uptake and Decarboxylation) System [4]. Characterized by amine and neuropeptide hormone production with dense vesicles, these neuroendocrine cells are specialized to receive neuronal inputs and consequentially release message peptides into circulation for the regulation and modulation of cell proliferation, growth, and development. NENs are distinguished from pheochromocytomas and paragangliomas (neuroendocrine non-epithelial neoplasms) by the expression of keratin in the former ones, given their epithelial origin [11].

Neuroendocrine tumors (NETs) represent only 0.5% of all malignant conditions and 2% of all malignant tumors in the gastrointestinal tract [12]. Given the continued update in the classification of NENs, these epidemiological data are continuously evolving. The prevalence of NETs ranges between 2.5 and 8.35 cases per 10,000, with a recent increase in their incidence rates [1–4,13–16], probably due to imaging improvement, leading to an earlier and more frequent diagnosis of the disease [6].

In the 2019 WHO classification of tumors of the digestive system [17], NENs are divided into well-differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs), based on their molecular differences. In addition, "mixed neuroendocrine–non-neuroendocrine neoplasms" (MiNENs) are better characterized, according to the simultaneous presence of both neuroendocrine and non-neuroendocrine components, typically poorly differentiated (Table 1).

Table 1. WHO classification (2019) and grading criteria for gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) [17].

	Differentiation	Grade	Mitotic Rate (Mitoses/2 mm^2)	Ki-67 Index
NET, G1		Low	<2	<3%
NET, G2	Well differentiated	Intermediate	2–20	3–20%
NET, G3		High	>20	>20%
NEC, small-cell type			>20	>20%
NEC, large-cell type	Poorly differentiated	High	>20	>20%
MiNEN	Well or poorly differentiated	Variable	Variable	Variable

The most frequent primary sites are the gastrointestinal tract (61%), lung (25%), and about 14% remains of an unknown origin [18]. A total of 12 to 22% of patients are metastatic at presentation [6].

Recently, abdominal pain was reported as an unspecific symptom of a small bowel NET [19]. Our case report resembled that very recently described by Daraghmeh et al. [19]; although, in our patient, we found a Pan-NET.

The 2019 WHO classification [17] provided an improved system for determining prognoses and treatments, appliable to all NENs, replacing the previous classification based on cell embryologic origin (foregut, midgut, and hindgut) [20]. In contrast to the 2017 WHO classification of tumors of endocrine organs [21], the last classification included pancreatic tumors in gastroenteropancreatic NENs (GEP-NENs) [17].

Gastroenteropancreatic tumors (GEP-NETs) are most commonly located in the gastric mucosa, the small intestine, the rectum, and the pancreas [4,22]. While a subset of NENs is functional (40%), presenting with characteristic endocrine-related symptoms, most of them are non-functional and do not present with symptoms until later stages.

The distant metastases of NF-PNETs are often found at the time of diagnosis, because symptoms of NF-PNETS develop in an advanced stage. Due to these characteristics, NF-PNETs are usually incidentally diagnosed, like GEP-NETS, thanks to the development of imaging techniques, able to also identify very small lesions. In our patient, the presence of flushing, diarrhea, and neuropsychiatric symptoms, suggesting carcinoid syndrome, was unrelated to a biochemical elevation of hormonal levels. As a matter of fact, small PNETs without metastases can often remain asymptomatic until they reach a significant dimension, or can present with unspecific symptoms, such as abdominal pain, weight loss, anorexia, and nausea.

Up to 90% of Pan-NETs are hormonally silent, a behavior affecting the prognosis as compared to functioning neoplasms, probably because of a late diagnosis [23].

Pan-NETs may produce a large variety of hormones, such as insulin, gastrin, glucagon, vasoactive intestinal peptide (VIP), serotonin, somatostatin, and others [24]. By contrast, non-functional Pan-NETs, without hormone overproduction, may present with unspecific symptoms, such as abdominal pain, weight loss, diarrhea, and gastrointestinal bleeding [8,25]. Most Pan-NENs are sporadic, whereas a minority are inherited, associated with type-1 multiple endocrine neoplasia (MEN-1), von Hippel–Lindau syndrome (VHL), tuberous sclerosis, or neurofibromatosis.

Functional pancreatic neuroendocrine tumors, associated with a variety of clinical syndromes, include [26] insulinomas, the most common functional Pan-NETs; gastrinomas, or Zollinger–Ellison syndrome; pancreatic polypeptide-secreting tumors; VIPomas, or Verner–Morrison syndrome; glucagonomas, exclusively localized in pancreas; and somatostatinomas, the least common NETs.

Carcinoid syndrome is a paraneoplastic syndrome that occurs because of the release of bio-active substances, predominantly serotonin (5-HT), but also histamine, bradykinin, prostaglandins E and F, and tachykinins [27]. The typical symptoms are flushing and

diarrhea. Wheezing, palpitations, breathlessness, abdominal pain, telangiectasias, and neuropsychiatric symptoms can also be associated with carcinoid syndrome [27,28]. Recently, Halperin et al. [29] demonstrated in a population-based analysis conducted on the American "Surveillance, Epidemiology, and End Results Medicare" database that 19% of patients with NETs had carcinoid syndrome. In patients harboring Pan-NETs, carcinoid syndrome is even more rare, accounting for approximately 1% [13].

The diagnosis of GEP-NENs is performed on the basis of a tissue histological examination [30]. Radiological and functional imaging is used to evaluate disease extension (staging) and assess the response to therapy, as well as to localize the primary site. Laboratory tests play a diagnostic role only in carcinoid syndrome and hormone-specific syndromes (gastrinomas, insulinomas, and glucagonomas), although the assay of either circulating or urinary hormones fail to be highly sensitive and specific, sometimes because blood sampling and urine collection are not performed closely to the occurrence of typical symptoms.

The current WHO classification emphasizes the role of histological examinations in surgically removed neoplasms, in order to establish the morphological characteristics and grading [17]. Three grades (G1, G2, and G3) are described for GEP-NETs, based on the proliferation activity assessed by the mitotic rate and Ki67 proliferation index [31,32]. For a more specific diagnosis, together with the morphology and grading, the immunohistochemical staining of chromogranin A (CgA) and synaptophysin should be assessed as biomarkers of neuroendocrine tumors.

Although the WHO histological classifications are specifically intended for surgically removed NENs [10,17], recent studies have investigated the role of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) and fine-needle biopsy (EUS-FNB) for the preoperative evaluation and management of pancreatic NETs (Pan-NETs) [33–42]. Despite the data for the grading agreement between EUS-FNA and surgical specimens highlighting the significant rate of under- or over-grading [41–47], the recent introduction of needles for EUS-guided fine-needle biopsies (EUS-FNBs), as for our patient, changed the scenario [37,38]. An EUS-FNB, in fact, allows us to obtain tissue samples on which immunohistochemical examinations can be easily performed, to evaluate the Ki67 proliferation index [39–45]. As a matter of fact, in patients harboring Pan-NETs smaller than 2 cm, the management remains still controversial, especially for asymptomatic and non-functional Pan-NETs [46–49]. Endoscopy with a biopsy is already the gold standard for diagnosing NENs of the stomach, duodenum, and colorectum [50,51]. In the diagnosis of pancreatic NENs, EUS is particularly useful in detecting the nature of small lesions. The introduction of EUS-FNB can then overcome the interpretative limits of EUS-FNA, therefore allowing the early characterization of tumors where surgery would destroy healthy tissue [39,40]. However, further prospective, randomized studies are needed to validate these approaches in the specific setting of Pan-NETs [30,52].

The surgical treatment of patients with small low-grade non-functional pancreatic neuroendocrine tumors (<20 mm) is still under debate, according to the ENETS guidelines. In this respect, Sugawara et al. [53], in a recent metanalysis, demonstrated that a surgical resection was recommended in patients with nonmetastatic NF-PNETs measuring between 1.1 and 2.0 cm; alternatively, those patients with a smaller lesion (<1 cm) showed greater prognostic benefits with a conservative approach. JNETS [54] suggests a follow-up strategy, with imaging every 6–12 months of asymptomatic tumors <1 cm without metastases. Moreover, Sadot et al. [55] further reported that, among 104 patients with small, asymptomatic Pan-NETs undergoing non-operative management, no patient developed evidence of metastases or died because of the tumor after a median follow up of 44 months. Several studies suggested that a surgical intervention may not be warranted for very small Pan-NETs, especially in elderly individuals [56–58]. It is noteworthy however that all these data were obtained from a population much younger (median age: 60–65 years) than our patient (81 years old).

Paik et al. [59] suggested that patients with Pan-NETs smaller than 1 cm could be managed by observation alone, while Pan-NETs > 1 cm should undergo EUS-FNBs to obtain grading and Ki67 immunostaining, to characterize the tumor according to the WHO classification.

To investigate Pan-NETs, several imaging techniques can be performed, including computed tomography (CT), magnetic resonance (MRI), ultrasonography, and functional imaging with scintigraphy and positron emission tomography (PET). The optimal choice of imaging modality depends on the location of primary and metastatic lesions [60].

Endoscopic ultrasonography (EUS) has become the gold standard technique to evaluate pancreatic neuroendocrine lesions [4,30,61,62]. On an EUS, Pan-NETs typically appear as well-defined, round, hypoechoic, homogenous vascular lesions [63]. As in our case report, the EUS allowed the accurate localization of Pan-NETs, which was crucial for surgical interventions. As mentioned before, the EUS allows the cyto-histological confirmation of neuroendocrine tumors through guided tissue acquisition for histological procedures [33–42].

The functional imaging of GEP-NENs is based on the typical expression of somatostatin receptors (SSTRs) by neuroendocrine cells [64]. In the past, functional studies were performed with [111]indium pentetreotide scintigraphy (Octreoscan[®]); in recent years, PET/CT with somatostatin analogs tracked with gallium-68 (^{68}Ga-SSA PET/CT) has become the modality of choice for SSTR imaging [10,65,66]. Functional imaging is indicated for staging, the localization of the unknown primary tumor in patients with established neuroendocrine metastases, the in vivo demonstration of SSTR expression in neuroendocrine cells (for therapeutic planning), as well as the extent of disease after treatment. The most common somatostatin analogs used in clinical practice are ^{68}Ga-DOTA-Tyr3-octreotide (^{68}Ga-DOTA-TOC), ^{68}Ga-DOTA-Tyr3-octreotate (^{68}Ga-DOTA-TATE), and ^{68}Ga-DOTA-Nal3-octreotide (^{68}Ga-DOTA-NOC). The mean sensitivity of ^{68}Ga-DOTA-SSA PET/CT for the diagnosis of Pan-NETs was 92%, while the specificity was 83% [67,68]. In advanced, fast-growing G2 and G3 NENs, especially if receptor negativity was evident at ^{68}Ga-SSA PET/CT, [18]FDG-PET/CT could be considered in the diagnostic approach [69,70]. The detection of Pan-NETs with functional imaging can be affected by the physiological uptake, especially in the uncinate process, therefore suggesting morphological imaging together with histological confirmation as a specific diagnostic process [71]. However, it still remains under debate whether the combined use of [18]FDG-PET/CT and the ^{68}Ga-DOTA-TOC peptide can improve the diagnostic performance of NENs [70]. Of note, a recent retrospective study [72] confirmed the suggestion of the combined use of 68Ga-DOTA peptides and 18F-FDG as radiotracers for a dual-tracer PET/CT to better evaluate tumor aggressiveness before surgery, especially for small masses of doubtful interpretation, when a metabolic confirmation of biopsy grading is needed [73].

At present, the biochemical diagnosis of NENs has been downsized due to the high proportion of non-functioning NENs. Considering the high rates of false-positive and heterogeneous serum determinations, chromogranin A (CgA) should be used in patients with an already documented diagnosis of NEN, in order to establish the treatment response or during the follow up [74,75]; although, the results are less sensitive for the primary diagnosis. On the other hand, neuron-specific enolase (NSE) is considered an unreliable diagnostic biomarker for NETs, due to its low sensitivity and specificity, while no evidence is available regarding its role in the follow up [76].

Laboratory tests for specific biomarkers (gastrin, insulin, glucagon, VIP, and 5-HIAA) are still important tools for certain clinical syndromes. 5-hydroxyindoleacetic acid (5-HIAA), detected in a 24 h urine collection using optimal conditions for the assay, is the specific tumor marker of carcinoid syndrome. 5-HIAA demonstrated a diagnostic sensitivity of 70%, with a specificity of 90% [77]. It is not recommended to use 5-HIAA as a screening test in the presence of diarrhea. Instead, it should be used in patients diagnosed with NENs to confirm carcinoid syndrome and assess its response to therapy [10,77].

Circulating tumor cells, circulating tumor DNA, circulating micro-RNAs, and NETest (the simultaneous measurement of 51 neuroendocrine-specific marker genes in the peripheral blood) are novel biomarkers under validation for NENs. However, this test is not widely available and needs further validation [78].

The treatment of Pan-NENs depends on the functionality, localization, dimension, and disease progression of the tumor. In most cases, surgical resection is the appropriate curative treatment in functioning pancreatic NET syndromes without metastases [49,54]. As for NF-Pan-NETs, surgical treatment, when feasible, is the gold standard [46,79,80], even if, as previously mentioned, a surgical intervention may not be warranted for very small Pan-NETs (<1.0 cm), especially in elderly individuals [53–58]. Surgical options include simple enucleation, central pancreatectomy, distal pancreatectomy with or without a splenectomy, and pancreatoduodenectomy (Whipple's operation), depending on the tumor's location. Moreover, radiofrequency ablation and trans-arterial chemoembolization are used for liver metastases.

When a macroscopic curative resection is unfeasible, medical treatment is indicated to control hormonal symptoms in F-Pan-NETs and to reduce the tumor's growth. Since the majority of GEP-NETs express somatostatin receptors (SSTRs), somatostatin analogs are used in F-Pan-NETs, together with adequate treatments for specific clinical syndromes (for example, PPi in ZES) [81]. For tumor growth control, somatostatin analogs, molecular-targeted drugs, and cytotoxic anticancer agents are indicated, regardless of functionality [82]. SSAs are the first choice when a positive expression of SSRT is confirmed. The use of lanreotide and octreotide long-acting release (LAR) were already proven to be effective in reducing a tumor's progression [81,83,84]. Recently, Wolin et al. [85] reported that the use of pasireotide, a novel SSA, despite a more extensive antiproliferation effect, was associated with more frequent adverse events. Targeted therapy, with everolimus and sunitinib, chemotherapy, and peptide-receptor radionuclide therapy (PRRT) should generally be reserved for SSA-refractory cases [49].

4. Discussion

Our case report described an old patient with an extremely rare pancreatic neuroendocrine tumor (Pan-NET), diagnosed in the presence of unspecific gastrointestinal symptoms and skin flushing. This observation is even rarer in old people. Despite the symptoms suggesting carcinoid syndrome, the tumor was well-differentiated and localized in the pancreas without liver metastases. This presentation is extremely rare, with few cases reported in the literature [86–88]. Biochemical testing for serum CgA and urinary 5-HIAA resulted negative. As previously emphasized, laboratory biomarkers were recently downsized due to the high rates of false positivity and their pharmacological interference, leading to low sensitivity and specificity [74–78,89].

We confirmed the Pan-NETs diagnosis through a contrast-enhanced CT, followed by functional imaging with a gallium-68 DOTATOC integrated PET/CT. We decided to perform an EUS-FNB to test immunostaining for the main markers of Pan-NETs and obtain grading. EUS-FNB confirmed the diagnosis of well-differentiated, low-grade (G1) Pan-NET (CgA+, Synaptophysin+, Ki67 1%).

The association of NETs and carcinoid syndrome occurs in approximately 19% of patients [27]. Except for patients with primary ovarian or bronchial neuroendocrine tumors, the evidence of carcinoid syndrome develops when metastases have occurred [26,28]. As a matter of fact, serotonin-producing Pan-NETs account for 0.58–1.4% of all Pan-NETs [90,91]. Only a few cases have been previously reported for Pan-NETs without liver metastases presenting with carcinoid syndrome [87,92]. Some patients with neuroendocrine tumors showed symptoms of flushing with low or normal levels of 5-HIAA [93,94]. Negativity for the immunostaining of serotonin found in our tumor biopsy, while in keeping with the normal values of 5HIAA, may further support the notion that levels of circulating hormones can increase only in the presence of liver metastases [29]. Our patient experienced carcinoid symptoms (diarrhea, flushing, and unresponsive depression) in the absence of

documented liver metastases and with negative serum CgA and normal urinary 5-HIAA levels. The guidelines clearly show that negative hormone measurements define NETs as "nonfunctional", even if presenting with suggestive symptoms or positive hormonal expressions in NET cells on immunohistochemical staining [49]. This may not always true, as can be observed in our case-report, as well as in few other reports [87,92].

It remains unclear why symptoms resembling carcinoid syndrome developed in our patient, with no evidence of any increase in hormone levels. It may well be that a possible, sudden, and transient hormone increase in the circulation failed to be detected. Otherwise, some to date unknown mechanisms might have been responsible for the abdominal pain, diarrhea, and flushing, all together causing us to consider alternative diagnoses regarding bowel diseases, which were excluded by the contrast-enhanced CT scan in our patient. In the presence of this discrepancy between the presence of symptoms and hormone negativity however, our case report emphasized that the clinical presentation should not be disregarded as a presentation of carcinoid-like syndrome, therefore leading to a complete diagnostic work-up for NETs.

Therefore, despite this, the Pan-NET of our patient should be defined as non-functional according to the guidelines [49], because the hormone values were within the normal range; our case report demonstrated that the imaging and histological examinations were useful in the diagnostic work-up of a Pan-NET associated with symptoms of carcinoid syndrome. As we reported, performing an EUS-FNB and assessing the cyto-histological features can help characterize the Pan-NET. Of note, we again underscore the concept that Pan-NET occurrence without metastases in old patients is very rare.

A Pan-NET < 1.0 cm can occur in very old people, without metastases, as in our case report; although, the median age was between 61 and 65 years in a recent metanalysis [50]. A surgical resection in these cases is not warranted. On the contrary, for Pan-NETs between 1.0 and 2.0 cm, surgical resections provided a better survival outcome, but in patients younger than 65 years old, without comorbidities.

The novelties of our case report can be highlighted as follows: (1) the symptoms of carcinoid syndrome can be shown in a Pan-NET < 1.0 cm occurring in very old people, without metastases, and with no evidence of an increase in circulating hormones, in agreement with the negativity of immunostaining for serotonin shown in tumor tissue. To date, the median age range was much lower [52]. (2) The categorical distinction of "functional" and "nonfunctional" NETs suggested by the guidelines [49] on the basis of hormone positivity and clinical presentation can help present Pan-NETs with no evidence of hormone release, as in our case report, thus underscoring the concept that the physician should take into account the possibility that an atypical pattern of apparently "non-functional" Pan-NETs may occur, although rarely. (3) In our patient, the EUS-FNB offered the opportunity of obtaining additional data regarding the immunostaining of the small Pan-NET; although, to date, an EUS-FNA is recognized as the method of choice in the multidisciplinary diagnostic approach of occult primary NETs, as recently reported by Rossi et al. [62]. Further evidence is needed to understand whether an EUS-FNB, as reported by a recent multicenter study [53], can provide physicians with additional details of the diagnostic workup of Pan-NETs.

In conclusion, this case report contributes to the understanding of the clinical spectrum of Pan-NETs, particularly in elderly patients, and highlights the potential challenges in decision making when treating patients with indolent neoplasms, as well as well-differentiated Pan-NETs surgically or even medicinally. It also highlights the role of advanced diagnostic techniques, such as EUS-FNB, in characterizing P-NETs.

Abbreviations

APUD	Amine precursor uptake and decarboxylation
CgA	Chromogranin A
CHD	Carcinoid heart disease
Cr	Creatinine
CT	Computed tomography
CRP	C-reactive protein
eGFR	Estimated glomerular filtration rate
ENETS	European neuroendocrine tumor society
EUS-FNA	Endoscopic ultrasound with fine-needle aspiration
EUS-FNB	Endoscopic ultrasound with fine-needle biopsy
FAST	Focused assessment with sonography in trauma
^{68}Ga-DOTA-NOC	Gallium-68-DOTA-Nal3-octreotide
^{68}Ga-DOTA-TATE	Gallium-68-DOTA-Tyr3-octreotate
^{68}Ga-DOTA-TOC	Gallium-68-DOTA-Tyr3-octreotide
GEP-NEN	Gastroenteropancreatic neuroendocrine neoplasm
GEP-NET	Gastroenteropancreatic neuroendocrine tumor
HGT	Hemo-glucose test
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
INSM1	Insulinoma-associated protein 1
JNETS	Japanese neuroendocrine tumor society
MEN-1	Multiple-endocrine neoplasia 1
MiNEN	Mixed neuroendocrine–non-neuroendocrine neoplasm
MRI	Magnetic-resonance imaging
NF-Pan-NET	Non-functional pancreatic neuroendocrine tumor
NEC	Neuroendocrine carcinoma
NEN	Neuroendocrine neoplasm
NET	Neuroendocrine tumor
NSE	Neuron-specific enolase
Pan-NET	Pancreatic neuroendocrine tumor
PAD	Peripheral artery disease
PET	Positive-emission tomography
PPi	Proton pomp inhibitor
PPRT	Peptide receptor radionuclide therapy
SaO2	Oxygen saturation
SSA	Somatostatin analog
SSTR	Somatostatin receptor
VHL	von Hippel–Lindau syndrome
VIP	Vaso-active intestinal peptide
WHO	World Health Organization

References

1. Yao, J.C.; Hassan, M.; Phan, A.; Dagohoy, C.; Leary, C.; Mares, J.E.; Abdalla, E.K.; Fleming, J.B.; Vauthey, J.N.; Rashid, A.; et al. One hundred years after "carcinoid": Epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **2008**, *26*, 3063–3072. [CrossRef] [PubMed]
2. Das, S.; Dasari, A. Epidemiology, Incidence, and Prevalence of Neuroendocrine Neoplasms: Are There Global Differences? *Curr. Oncol. Rep.* **2021**, *23*, 43. [CrossRef] [PubMed]
3. Leoncini, E.; Boffetta, P.; Shafir, M.; Aleksovska, K.; Boccia, S.; Rindi, G. Increased incidence trend of low-grade and high-grade neuroendocrine neoplasms. *Endocrine* **2017**, *58*, 368–379. [CrossRef] [PubMed]
4. Rossi, R.E.; Massironi, S. The Increasing Incidence of Neuroendocrine Neoplasms Worldwide: Current Knowledge and Open Issues. *J. Clin. Med.* **2022**, *11*, 3794. [CrossRef]
5. Pavel, M.; Öberg, K.; Falconi, M.; Krenning, E.P.; Sundin, A.; Perren, A.; Berruti, A.; ESMO Guidelines Committee. Gastroenteropancreatic neuroendocrine neoplasms: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* **2020**, *31*, 844–860. [CrossRef]
6. Dasari, A.; Shen, C.; Halperin, D.; Zhao, B.; Zhou, S.; Xu, Y.; Shih, T.; Yao, J.C. Trends in the Incidence, Prevalence, and Survival Outcomes in Patients with Neuroendocrine Tumors in the United States. *JAMA Oncol.* **2017**, *3*, 1335–1342. [CrossRef] [PubMed]
7. Clement, D.; Ramage, J.; Srirajaskanthan, R. Update on Pathophysiology, Treatment, and Complications of Carcinoid Syndrome. *J. Oncol.* **2020**, *2020*, 8341426. [CrossRef]
8. Metz, D.C.; Jensen, R.T. Gastrointestinal neuroendocrine tumors: Pancreatic endocrine tumors. *Gastroenterology* **2008**, *135*, 1469–1492. [CrossRef]
9. Garcia-Carbonero, R.; Capdevila, J.; Crespo-Herrero, G.; Díaz-Pérez, J.A.; Martínez Del Prado, M.P.; Alonso Orduña, V.; Sevilla-García, I.; Villabona-Artero, C.; Beguiristain-Gómez, A.; Llanos-Muñoz, M.; et al. Incidence, patterns of care and prognostic factors for outcome of gastroenteropancreatic neuroendocrine tumors (GEP-NETs): Results from the National Cancer Registry of Spain (RGETNE). *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol.* **2010**, *21*, 1794–1803. [CrossRef]
10. AIOM for Neuroendocrine Tumors Guide Line. 2020. Available online: https://www.aiom.it/wp-content/uploads/2020/10/2020_LG_AIOM_Neuroendocrini.pdf (accessed on 15 July 2023).
11. Ordóñez, N.G. Broad-spectrum immunohistochemical epithelial markers: A review. *Hum. Pathol.* **2013**, *44*, 1195–1215. [CrossRef]
12. Moertel, C.G. Karnofsky memorial lecture. An odyssey in the land of small tumors. *J. Clin. Oncol.* **1987**, *5*, 1502–1522. [CrossRef]
13. Ito, T.; Igarashi, H.; Nakamura, K.; Sasano, H.; Okusaka, T.; Takano, K.; Komoto, I.; Tanaka, M.; Imamura, M.; Jensen, R.T.; et al. Epidemiological trends of pancreatic and gastrointestinal neuroendocrine tumors in Japan: A nationwide survey analysis. *J. Gastroenterol.* **2015**, *50*, 58–64. [CrossRef] [PubMed]
14. Cho, M.Y.; Kim, J.M.; Sohn, J.H.; Kim, M.J.; Kim, K.M.; Kim, W.H.; Kim, H.; Kook, M.C.; Park, D.Y.; Lee, J.H.; et al. Current Trends of the Incidence and Pathological Diagnosis of Gastroenteropancreatic Neuroendocrine Tumors (GEP-NETs) in Korea 2000–2009: Multicenter Study. *Cancer Res. Treat.* **2012**, *44*, 157–165. [CrossRef]
15. Scherübl, H.; Streller, B.; Stabenow, R.; Herbst, H.; Höpfner, M.; Schwertner, C.; Steinberg, J.; Eick, J.; Ring, W.; Tiwari, K.; et al. Clinically detected gastroenteropancreatic neuroendocrine tumors are on the rise: Epidemiological changes in Germany. *World J. Gastroenterol.* **2013**, *19*, 9012–9019. [CrossRef] [PubMed]
16. White, B.E.; Rous, B.; Chandrakumaran, K.; Wong, K.; Bouvier, C.; Van Hemelrijck, M.; George, G.; Russell, B.; Srirajaskanthan, R.; Ramage, J.K. Incidence and survival of neuroendocrine neoplasia in England 1995–2018: A retrospective, population-based study. *Lancet Reg. Health. Eur.* **2022**, *23*, 100510. [CrossRef] [PubMed]
17. Nagtegaal, I.D.; Odze, R.D.; Klimstra, D.; Paradis, V.; Rugge, M.; Schirmacher, P.; Washington, K.M.; Carneiro, F.; Cree, I.A.; WHO Classification of Tumours Editorial Board. The 2019 WHO classification of tumours of the digestive system. *Histopathology* **2020**, *76*, 182–188. [CrossRef]
18. Fernandez, C.J.; Agarwal, M.; Pottakkat, B.; Haroon, N.N.; George, A.S.; Pappachan, J.M. Gastroenteropancreatic neuroendocrine neoplasms: A clinical snapshot. *World J. Gastrointest. Surg.* **2021**, *13*, 231–255. [CrossRef]
19. Daraghmeh, L.; Shbaita, S.; Nassef, O.; Melhem, L.; Maqboul, I. Non-specific Symptoms of Small Bowel Neuroendocrine Tumor and the Diagnostic Challenges: A Case Report. *Cureus* **2023**, *15*, e41080. [CrossRef]
20. Klöppel, G.; Perren, A.; Heitz, P.U. The gastroenteropancreatic neuroendocrine cell system and its tumors: The WHO classification. *Ann. N. Y. Acad. Sci.* **2004**, *1014*, 13–27. [CrossRef]
21. Lloyd, R.V.; Osamura, R.Y.; Klöppel, G.; Rosai, J. *WHO Classification of Tumours of Endocrine Organs*, 4th ed.; International Agency for Research on Cancer: Lyon, France, 2017; Volume 10.
22. Modlin, I.M.; Lye, K.D.; Kidd, M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* **2003**, *97*, 934–959. [CrossRef]
23. Cives, M.; Strosberg, J.R. Gastroenteropancreatic neuroendocrine tumors. *CA A Cancer J. Clin.* **2018**, *68*, 471–487. [CrossRef] [PubMed]
24. Turaga, K.K.; Kvols, L.K. Recent progress in the understanding, diagnosis, and treatment of gastroenteropancreatic neuroendocrine tumors. *CA Cancer J. Clin.* **2011**, *61*, 113–132. [CrossRef]
25. Zerbi, A.; Falconi, M.; Rindi, G.; Delle Fave, G.; Tomassetti, P.; Pasquali, C.; Capitanio, V.; Boninsegna, L.; Di Carlo, V.; AISP-Network Study Group. Clinicopathological features of pancreatic endocrine tumors: A prospective multicenter study in Italy of 297 sporadic cases. *Am. J. Gastroenterol.* **2010**, *105*, 1421–1429. [CrossRef]

26. Jensen, R.T.; Cadiot, G.; Brandi, M.L.; de Herder, W.W.; Kaltsas, G.; Komminoth, P.; Scoazec, J.Y.; Salazar, R.; Sauvanet, A.; Kianmanesh, R.; et al. ENETS Consensus Guidelines for the management of patients with digestive neuroendocrine neoplasms: Functional pancreatic endocrine tumor syndromes. *Neuroendocrinology* **2012**, *95*, 98–119. [CrossRef]

27. Gade, A.K.; Olariu, E.; Douthit, N.T. Carcinoid Syndrome: A Review. *Cureus* **2020**, *12*, e7186. [CrossRef]

28. Mota, J.M.; Sousa, L.G.; Riechelmann, R.P. Complications from carcinoid syndrome: Review of the current evidence. *Ecancermedicalscience* **2016**, *10*, 662. [CrossRef]

29. Halperin, D.M.; Shen, C.; Dasari, A.; Xu, Y.; Chu, Y.; Zhou, S.; Shih, Y.T.; Yao, J.C. Frequency of carcinoid syndrome at neuroendocrine tumour diagnosis: A population-based study. *Lancet. Oncol.* **2017**, *18*, 525–534. [CrossRef]

30. Rossi, R.E.; Corti, F.; Pusceddu, S.; Milione, M.; Coppa, J.; Masoni, B.; Oldani, S.; Sabella, G.; Cafaro, P.; Repici, A. Multidisciplinary Approach to the Diagnosis of Occult Primary Neuroendocrine Neoplasm: A Clinical Challenge. *J. Clin. Med.* **2023**, *12*, 5537. [CrossRef]

31. Tatsumoto, S.; Kodama, Y.; Sakurai, Y.; Shinohara, T.; Katanuma, A.; Maguchi, H. Pancreatic neuroendocrine neoplasm: Correlation between computed tomography enhancement patterns and prognostic factors of surgical and endoscopic ultrasound-guided fine-needle aspiration biopsy specimens. *Abdom. Imaging* **2013**, *38*, 358–366. [CrossRef] [PubMed]

32. Grillo, F.; Albertelli, M.; Brisigotti, M.P.; Borra, T.; Boschetti, M.; Fiocca, R.; Ferone, D.; Mastracci, L. Grade Increases in Gastroenteropancreatic Neuroendocrine Tumor Metastases Compared to the Primary Tumor. *Neuroendocrinology* **2016**, *103*, 452–459. [CrossRef] [PubMed]

33. Atiq, M.; Bhutani, M.S.; Bektas, M.; Lee, J.E.; Gong, Y.; Tamm, E.P.; Shah, C.P.; Ross, W.A.; Yao, J.; Raju, G.S.; et al. EUS-FNA for pancreatic neuroendocrine tumors: A tertiary cancer center experience. *Dig. Dis. Sci.* **2012**, *57*, 791–800. [CrossRef] [PubMed]

34. Larghi, A.; Capurso, G.; Carnuccio, A.; Ricci, R.; Alfieri, S.; Galasso, D.; Lugli, F.; Bianchi, A.; Panzuto, F.; De Marinis, L.; et al. Ki-67 grading of nonfunctioning pancreatic neuroendocrine tumors on histologic samples obtained by EUS-guided fine-needle tissue acquisition: A prospective study. *Gastrointest. Endosc.* **2012**, *76*, 570–577. [CrossRef] [PubMed]

35. Unno, J.; Kanno, A.; Masamune, A.; Kasajima, A.; Fujishima, F.; Ishida, K.; Hamada, S.; Kume, K.; Kikuta, K.; Hirota, M.; et al. The usefulness of endoscopic ultrasound-guided fine-needle aspiration for the diagnosis of pancreatic neuroendocrine tumors based on the World Health Organization classification. *Scand. J. Gastroenterol.* **2014**, *49*, 1367–1374. [CrossRef]

36. Paiella, S.; Landoni, L.; Rota, R.; Valenti, M.; Elio, G.; Crinò, S.F.; Manfrin, E.; Parisi, A.; Cingarlini, S.; D'Onofrio, M.; et al. Endoscopic ultrasound-guided fine-needle aspiration for the diagnosis and grading of pancreatic neuroendocrine tumors: A retrospective analysis of 110 cases. *Endoscopy* **2020**, *52*, 988–994. [CrossRef] [PubMed]

37. Crinò, S.F.; Ammendola, S.; Meneghetti, A.; Bernardoni, L.; Conti Bellocchi, M.C.; Gabbrielli, A.; Landoni, L.; Paiella, S.; Pin, F.; Parisi, A.; et al. Comparison between EUS-guided fine-needle aspiration cytology and EUS-guided fine-needle biopsy histology for the evaluation of pancreatic neuroendocrine tumors. *Pancreatology* **2021**, *21*, 443–450. [CrossRef]

38. Rimbaş, M.; Crino, S.F.; Gasbarrini, A.; Costamagna, G.; Scarpa, A.; Larghi, A. EUS-guided fine-needle tissue acquisition for solid pancreatic lesions: Finally moving from fine-needle aspiration to fine-needle biopsy? *Endosc. Ultrasound* **2018**, *7*, 137–140. [CrossRef]

39. Eusebi, L.H.; Thorburn, D.; Toumpanakis, C.; Frazzoni, L.; Johnson, G.; Vessal, S.; Luong, T.V.; Caplin, M.; Pereira, S.P. Endoscopic ultrasound-guided fine-needle aspiration vs fine-needle biopsy for the diagnosis of pancreatic neuroendocrine tumors. *Endosc. Int. Open* **2019**, *7*, E1393–E1399. [CrossRef]

40. Di Leo, M.; Poliani, L.; Rahal, D.; Auriemma, F.; Anderloni, A.; Ridolfi, C.; Spaggiari, P.; Capretti, G.; Di Tommaso, L.; Preatoni, P.; et al. Pancreatic Neuroendocrine Tumours: The Role of Endoscopic Ultrasound Biopsy in Diagnosis and Grading Based on the WHO 2017 Classification. *Dig. Dis.* **2019**, *37*, 325–333. [CrossRef]

41. Leeds, J.S.; Nayar, M.K.; Bekkali, N.L.H.; Wilson, C.H.; Johnson, S.J.; Haugk, B.; Darne, A.; Oppong, K.W. Endoscopic ultrasound-guided fine-needle biopsy is superior to fine-needle aspiration in assessing pancreatic neuroendocrine tumors. *Endosc. Int. Open* **2019**, *7*, E1281–E1287. [CrossRef]

42. Melita, G.; Pallio, S.; Tortora, A.; Crinò, S.F.; Macrì, A.; Dionigi, G. Diagnostic and Interventional Role of Endoscopic Ultrasonography for the Management of Pancreatic Neuroendocrine Neoplasms. *J. Clin. Med.* **2021**, *10*, 2638. [CrossRef]

43. Bernstein, J.; Ustun, B.; Alomari, A.; Bao, F.; Aslanian, H.R.; Siddiqui, U.; Chhieng, D.; Cai, G. Performance of endoscopic ultrasound-guided fine needle aspiration in diagnosing pancreatic neuroendocrine tumors. *CytoJournal* **2013**, *10*, 10. [CrossRef]

44. Boutsen, L.; Jouret-Mourin, A.; Borbath, I.; van Maanen, A.; Weynand, B. Accuracy of Pancreatic Neuroendocrine Tumour Grading by Endoscopic Ultrasound-Guided Fine Needle Aspiration: Analysis of a Large Cohort and Perspectives for Improvement. *Neuroendocrinology* **2018**, *106*, 158–166. [CrossRef] [PubMed]

45. Heidsma, C.M.; Tsilimigras, D.I.; Rocha, F.; Abbott, D.E.; Fields, R.; Smith, P.M.; Poultsides, G.A.; Cho, C.; van Eijck, C.; van Dijkum, E.N.; et al. Clinical relevance of performing endoscopic ultrasound-guided fine-needle biopsy for pancreatic neuroendocrine tumors less than 2 cm. *J. Surg. Oncol.* **2020**, *122*, 1393–1400. [CrossRef] [PubMed]

46. Gratian, L.; Pura, J.; Dinan, M.; Roman, S.; Reed, S.; Sosa, J.A. Impact of extent of surgery on survival in patients with small nonfunctional pancreatic neuroendocrine tumors in the United States. *Ann. Surg. Oncol.* **2014**, *21*, 3515–3521. [CrossRef] [PubMed]

47. Sharpe, S.M.; In, H.; Winchester, D.J.; Talamonti, M.S.; Baker, M.S. Surgical resection provides an overall survival benefit for patients with small pancreatic neuroendocrine tumors. *J. Gastrointest. Surg.* **2015**, *19*, 117–123. [CrossRef] [PubMed]

48. Sallinen, V.; Haglund, C.; Seppänen, H. Outcomes of resected nonfunctional pancreatic neuroendocrine tumors: Do size and symptoms matter? *Surgery* **2015**, *158*, 1556–1563. [CrossRef]
49. Hofland, J.; Falconi, M.; Christ, E.; Castaño, J.P.; Faggiano, A.; Lamarca, A.; Perren, A.; Petrucci, S.; Prasad, V.; Ruszniewski, P.; et al. European Neuroendocrine Tumor Society 2023 guidance paper for functioning pancreatic neuroendocrine tumour syndromes. *J. Neuroendocrinol.* **2023**, *35*, e13318. [CrossRef]
50. Delle Fave, G.; O'Toole, D.; Sundin, A.; Taal, B.; Ferolla, P.; Ramage, J.K.; Ferone, D.; Ito, T.; Weber, W.; Zheng-Pei, Z.; et al. ENETS Consensus Guidelines Update for Gastroduodenal Neuroendocrine Neoplasms. *Neuroendocrinology* **2016**, *103*, 119–124. [CrossRef]
51. Ramage, J.K.; De Herder, W.W.; Delle Fave, G.; Ferolla, P.; Ferone, D.; Ito, T.; Ruszniewski, P.; Sundin, A.; Weber, W.; Zheng-Pei, Z.; et al. ENETS Consensus Guidelines Update for Colorectal Neuroendocrine Neoplasms. *Neuroendocrinology* **2016**, *103*, 139–143. [CrossRef]
52. Delconte, G.; Cavalcoli, F.; Magarotto, A.; Centonze, G.; Bezzio, C.; Cattaneo, L.; Rausa, E.; Kelly, M.E.; Bonitta, G.; Milione, M.; et al. Does ProCore Fine-Needle Biopsy Really Improve the Clinical Outcome of Endoscopic Ultrasound-Guided Sampling of Pancreatic Masses? *Dig. Dis.* **2022**, *40*, 78–84. [CrossRef]
53. Sugawara, T.; Rodriguez Franco, S.; Kirsch, M.J.; Colborn, K.L.; Ishida, J.; Grandi, S.; Al-Musawi, M.H.; Gleisner, A.; Del Chiaro, M.; Schulick, R.D. Evaluation of Survival Following Surgical Resection for Small Nonfunctional Pancreatic Neuroendocrine Tumors. *JAMA Netw. Open* **2023**, *6*, e234096. [CrossRef]
54. Ito, T.; Masui, T.; Komoto, I.; Doi, R.; Osamura, R.Y.; Sakurai, A.; Ikeda, M.; Takano, K.; Igarashi, H.; Shimatsu, A.; et al. JNETS clinical practice guidelines for gastroenteropancreatic neuroendocrine neoplasms: Diagnosis, treatment, and follow-up: A synopsis. *J. Gastroenterol.* **2021**, *56*, 1033–1044. [PubMed]
55. Sadot, E.; Reidy-Lagunes, D.L.; Tang, L.H.; Do, R.K.; Gonen, M.; D'Angelica, M.I.; DeMatteo, R.P.; Kingham, T.P.; Groot Koerkamp, B.; Untch, B.R.; et al. Observation versus Resection for Small Asymptomatic Pancreatic Neuroendocrine Tumors: A Matched Case-Control Study. *Ann. Surg. Oncol.* **2016**, *23*, 1361–1370. [CrossRef]
56. Massironi, S.; Rossi, R.E.; Zilli, A.; Casazza, G.; Ciafardini, C.; Conte, D. A wait-and-watch approach to small pancreatic neuroendocrine tumors: Prognosis and survival. *Oncotarget* **2016**, *7*, 18978–18983. [CrossRef] [PubMed]
57. Partelli, S.; Ramage, J.K.; Massironi, S.; Zerbi, A.; Kim, H.B.; Niccoli, P.; Panzuto, F.; Landoni, L.; Tomazic, A.; Ibrahim, T.; et al. Management of Asymptomatic Sporadic Nonfunctioning Pancreatic Neuroendocrine Neoplasms (ASPEN) ≤ 2 cm: Study Protocol for a Prospective Observational Study. *Front. Med.* **2020**, *7*, 598438. [CrossRef] [PubMed]
58. Partelli, S.; Massironi, S.; Zerbi, A.; Niccoli, P.; Kwon, W.; Landoni, L.; Panzuto, F.; Tomazic, A.; Bongiovanni, A.; Kaltsas, G.; et al. Management of asymptomatic sporadic non-functioning pancreatic neuroendocrine neoplasms no larger than 2 cm: Interim analysis of prospective ASPEN trial. *Br. J. Surg.* **2022**, *109*, 1186–1190. [CrossRef]
59. Paik, W.H.; Lee, H.S.; Lee, K.J.; Jang, S.I.; Lee, W.J.; Hwang, J.H.; Cho, C.M.; Park, C.H.; Han, J.; Woo, S.M.; et al. Malignant potential of small pancreatic neuroendocrine neoplasm and its risk factors: A multicenter nationwide study. *Pancreatology* **2021**, *21*, 208–214. [CrossRef]
60. Morse, B.; Al-Toubah, T.; Montilla-Soler, J. Anatomic and functional imaging of neuroendocrine tumors. *Curr. Treat. Options Oncol.* **2020**, *21*, 75. [CrossRef]
61. Zilli, A.; Arcidiacono, P.G.; Conte, D.; Massironi, S. Clinical impact of endoscopic ultrasonography on the management of neuroendocrine tumors: Lights and shadows. *Dig. Liver Dis. Off. J. Ital. Soc. Gastroenterol. Ital. Assoc. Study Liver* **2018**, *50*, 6–14. [CrossRef]
62. Rossi, R.E.; Elvevi, A.; Gallo, C.; Palermo, A.; Invernizzi, P.; Massironi, S. Endoscopic techniques for diagnosis and treatment of gastro-entero-pancreatic neuroendocrine neoplasms: Where we are. *World J. Gastroenterol.* **2022**, *28*, 3258–3273. [CrossRef]
63. Lee, D.W.; Kim, M.K.; Kim, H.G. Diagnosis of Pancreatic Neuroendocrine Tumors. *Clin. Endosc.* **2017**, *50*, 537–545. [CrossRef]
64. Rust, E.; Hubele, F.; Marzano, E.; Goichot, B.; Pessaux, P.; Kurtz, J.E.; Imperiale, A. Nuclear medicine imaging of gastro-entero-pancreatic neuroendocrine tumors. The key role of cellular differentiation and tumor grade: From theory to clinical practice. *Cancer Imaging* **2012**, *12*, 173–184. [CrossRef] [PubMed]
65. Buchmann, I.; Henze, M.; Engelbrecht, S.; Eisenhut, M.; Runz, A.; Schäfer, M.; Schilling, T.; Haufe, S.; Herrmann, T.; Haberkorn, U. Comparison of 68Ga-DOTATOC PET and 111In-DTPAOC (Octreoscan) SPECT in patients with neuroendocrine tumours. *Eur. J. Nucl. Med. Mol. Imaging* **2007**, *34*, 1617–1626. [CrossRef] [PubMed]
66. Van Binnebeek, S.; Vanbilloen, B.; Baete, K.; Terwinghe, C.; Koole, M.; Mottaghy, F.M.; Clement, P.M.; Mortelmans, L.; Bogaerts, K.; Haustermans, K.; et al. Comparison of diagnostic accuracy of (111)In-pentetreotide SPECT and (68)Ga-DOTATOC PET/CT: A lesion-by-lesion analysis in patients with metastatic neuroendocrine tumours. *Eur. Radiol.* **2016**, *26*, 900–909. [CrossRef] [PubMed]
67. Treglia, G.; Castaldi, P.; Rindi, G.; Giordano, A.; Rufini, V. Diagnostic performance of Gallium-68 somatostatin receptor PET and PET/CT in patients with thoracic and gastroenteropancreatic neuroendocrine tumours: A meta-analysis. *Endocrine* **2012**, *42*, 80–87. [CrossRef]
68. Bozkurt, M.F.; Virgolini, I.; Balogova, S.; Beheshti, M.; Rubello, D.; Decristoforo, C.; Ambrosini, V.; Kjaer, A.; Delgado-Bolton, R.; Kunikowska, J.; et al. Guideline for PET/CT imaging of neuroendocrine neoplasms with ^{68}Ga-DOTA-conjugated somatostatin receptor targeting peptides and ^{18}F-DOPA. *Eur. J. Nucl. Med. Mol. Imaging* **2017**, *44*, 1588–1601. [CrossRef]
69. Binderup, T.; Knigge, U.; Loft, A.; Federspiel, B.; Kjaer, A. 18F-fluorodeoxyglucose positron emission tomography predicts survival of patients with neuroendocrine tumors. *Clin. Cancer Res.* **2010**, *16*, 978–985. [CrossRef]

70. Muffatti, F.; Partelli, S.; Cirocchi, R.; Andreasi, V.; Mapelli, P.; Picchio, M.; Gianolli, L.; Falconi, M. Combined 68Ga-DOTA-peptides and 18F-FDG PET in the diagnostic work-up of neuroendocrine neoplasms (NEN). *Clin. Transl. Imaging* **2019**, *7*, 181–188. [CrossRef]

71. Mapelli, P.; Tam, H.H.; Sharma, R.; Aboagye, E.O.; Al-Nahhas, A. Frequency and significance of physiological versus pathological uptake of 68Ga-DOTATATE in the pancreas: Validation with morphological imaging. *Nucl. Med. Commun.* **2014**, *35*, 613–619. [CrossRef]

72. Mapelli, P.; Partelli, S.; Salgarello, M.; Doraku, J.; Muffatti, F.; Schiavo Lena, M.; Pasetto, S.; Bezzi, C.; Bettinardi, V.; Andreasi, V.; et al. Dual Tracer 68Ga-DOTATOC and 18F-FDG PET Improve Preoperative Evaluation of Aggressiveness in Resectable Pancreatic Neuroendocrine Neoplasms. *Diagnostics* **2021**, *11*, 192. [CrossRef]

73. Paiella, S.; Landoni, L.; Tebaldi, S.; Zuffante, M.; Salgarello, M.; Cingarlini, S.; D'Onofrio, M.; Parisi, A.; Deiro, G.; Manfrin, E.; et al. Dual-Tracer (68Ga-DOTATOC and 18F-FDG-)-PET/CT Scan and G1-G2 Nonfunctioning Pancreatic Neuroendocrine Tumors: A Single-Center Retrospective Evaluation of 124 Nonmetastatic Resected Cases. *Neuroendocrinology* **2022**, *112*, 143–152. [CrossRef]

74. Leon, A.; Torta, M.; Dittadi, R.; degli Uberti, E.; Ambrosio, M.R.; Delle Fave, G.; De Braud, F.; Tomassetti, P.; Gion, M.; Dogliotti, L. Comparison between two methods in the determination of circulating chromogranin A in neuroendocrine tumors (NETs): Results of a prospective multicenter observational study. *Int. J. Biol. Markers* **2005**, *20*, 156–168. [CrossRef] [PubMed]

75. Welin, S.; Stridsberg, M.; Cunningham, J.; Granberg, D.; Skogseid, B.; Oberg, K.; Eriksson, B.; Janson, E.T. Elevated plasma chromogranin A is the first indication of recurrence in radically operated midgut carcinoid tumors. *Neuroendocrinology* **2009**, *89*, 302–307. [CrossRef]

76. Sansone, A.; Lauretta, R.; Vottari, S.; Chiefari, A.; Barnabei, A.; Romanelli, F.; Appetecchia, M. Specific and Non-Specific Biomarkers in Neuroendocrine Gastroenteropancreatic Tumors. *Cancers* **2019**, *11*, 1113. [CrossRef]

77. Oberg, K.; Couvelard, A.; Delle Fave, G.; Gross, D.; Grossman, A.; Jensen, R.T.; Pape, U.F.; Perren, A.; Rindi, G.; Ruszniewski, P.; et al. ENETS Consensus Guidelines for Standard of Care in Neuroendocrine Tumours: Biochemical Markers. *Neuroendocrinology* **2017**, *105*, 201–211. [CrossRef] [PubMed]

78. Ma, Z.Y.; Gong, Y.F.; Zhuang, H.K.; Zhou, Z.X.; Huang, S.Z.; Zou, Y.P.; Huang, B.W.; Sun, Z.H.; Zhang, C.Z.; Tang, T.Q.; et al. Pancreatic neuroendocrine tumors: A review of serum biomarkers, staging, and management. *World J. Gastroenterol.* **2020**, *26*, 2305–2322. [CrossRef]

79. Kulke, M.H.; Anthony, L.B.; Bushnell, D.L.; de Herder, W.W.; Goldsmith, S.J.; Klimstra, D.S.; Marx, S.J.; Pasieka, J.L.; Pommier, R.F.; Yao, J.C.; et al. NANETS treatment guidelines: Well-differentiated neuroendocrine tumors of the stomach and pancreas. *Pancreas* **2010**, *39*, 735–752. [CrossRef]

80. Knigge, U.; Hansen, C.P. Surgery for GEP-NETs. *Best Pract. Res. Clin. Gastroenterol.* **2012**, *26*, 819–831. [CrossRef]

81. Massironi, S.; Conte, D.; Rossi, R.E. Somatostatin analogues in functioning gastroenteropancreatic neuroendocrine tumours: Literature review, clinical recommendations and schedules. *Scand. J. Gastroenterol.* **2016**, *51*, 513–523. [CrossRef]

82. Pavel, M.; O'Toole, D.; Costa, F.; Capdevila, J.; Gross, D.; Kianmanesh, R.; Krenning, E.; Knigge, U.; Salazar, R.; Pape, U.F.; et al. ENETS Consensus Guidelines Update for the Management of Distant Metastatic Disease of Intestinal, Pancreatic, Bronchial Neuroendocrine Neoplasms (NEN) and NEN of Unknown Primary Site. *Neuroendocrinology* **2016**, *103*, 172–185. [CrossRef] [PubMed]

83. Caplin, M.E.; Pavel, M.; Ruszniewski, P. Lanreotide in metastatic enteropancreatic neuroendocrine tumors. *N. Engl. J. Med.* **2014**, *371*, 1556–1557. [CrossRef] [PubMed]

84. Jann, H.; Denecke, T.; Koch, M.; Pape, U.F.; Wiedenmann, B.; Pavel, M. Impact of octreotide long-acting release on tumour growth control as a first-line treatment in neuroendocrine tumours of pancreatic origin. *Neuroendocrinology* **2013**, *98*, 137–143. [CrossRef]

85. Wolin, E.M.; Jarzab, B.; Eriksson, B.; Walter, T.; Toumpanakis, C.; Morse, M.A.; Tomassetti, P.; Weber, M.M.; Fogelman, D.R.; Ramage, J.; et al. Phase III study of pasireotide long-acting release in patients with metastatic neuroendocrine tumors and carcinoid symptoms refractory to available somatostatin analogues. *Drug Des. Dev. Ther.* **2015**, *9*, 5075–5086. [CrossRef]

86. Feldman, J.M.; Jones, R.S. Carcinoid syndrome from gastrointestinal carcinoids without liver metastasis. *Ann. Surg.* **1982**, *196*, 33–37. [CrossRef] [PubMed]

87. Zavras, N.; Schizas, D.; Machairas, N.; Damaskou, V.; Economopoulos, N.; Machairas, A. Carcinoid syndrome from a carcinoid tumor of the pancreas without liver metastases: A case report and literature review. *Oncol. Lett.* **2017**, *13*, 2373–2376. [CrossRef]

88. Famerée, L.; Van Lier, C.; Borbath, I.; Yildiz, H.; Lemaire, J.; Baeck, M. Misleading clinical presentation of carcinoid syndrome. *Acta Gastro-Enterol. Belg.* **2021**, *84*, 501–503. [CrossRef]

89. Rossi, R.E.; Ciafardini, C.; Sciola, V.; Conte, D.; Massironi, S. Chromogranin A in the Follow-up of Gastroenteropancreatic Neuroendocrine Neoplasms: Is It Really Game Over? A Systematic Review and Meta-analysis. *Pancreas* **2018**, *47*, 1249–1255. [CrossRef]

90. Zandee, W.T.; van Adrichem, R.C.; Kamp, K.; Feelders, R.A.; van Velthuysen, M.F.; de Herder, W.W. Incidence and prognostic value of serotonin secretion in pancreatic neuroendocrine tumours. *Clin. Endocrinol.* **2017**, *87*, 165–170. [CrossRef] [PubMed]

91. Depoilly, T.; Leroux, R.; Andrade, D.; Nicolle, R.; Dioguardi Burgio, M.; Marinoni, I.; Dokmak, S.; Ruszniewski, P.; Hentic, O.; Paradis, V.; et al. Immunophenotypic and molecular characterization of pancreatic neuroendocrine tumors producing serotonin. *Modern pathology* **2022**, *35*, 1713–1722. [CrossRef]

92. Haq, A.U.; Yook, C.R.; Hiremath, V.; Kasimis, B.S. Carcinoid syndrome in the absence of liver metastasis: A case report and review of literature. *Med. Pediatr. Oncol.* **1992**, *20*, 221–223. [CrossRef]

93. Oberg, K.; Janson, E.T.; Eriksson, B. Tumour markers in neuroendocrine tumours. *Ital. J. Gastroenterol. Hepatol.* **1999**, *31* (Suppl. S2), S160–S162. [PubMed]

94. Eriksson, B.; Oberg, K.; Stridsberg, M. Tumor markers in neuroendocrine tumors. *Digestion* **2000**, *62* (Suppl. S1), 33–38. [CrossRef] [PubMed]

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