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Functional neuroendocrine tumors

Edited by
Bojana Popovic, Aviral Singh and Francesca Spada

Coordinated by
Karin Zibar Tomsic

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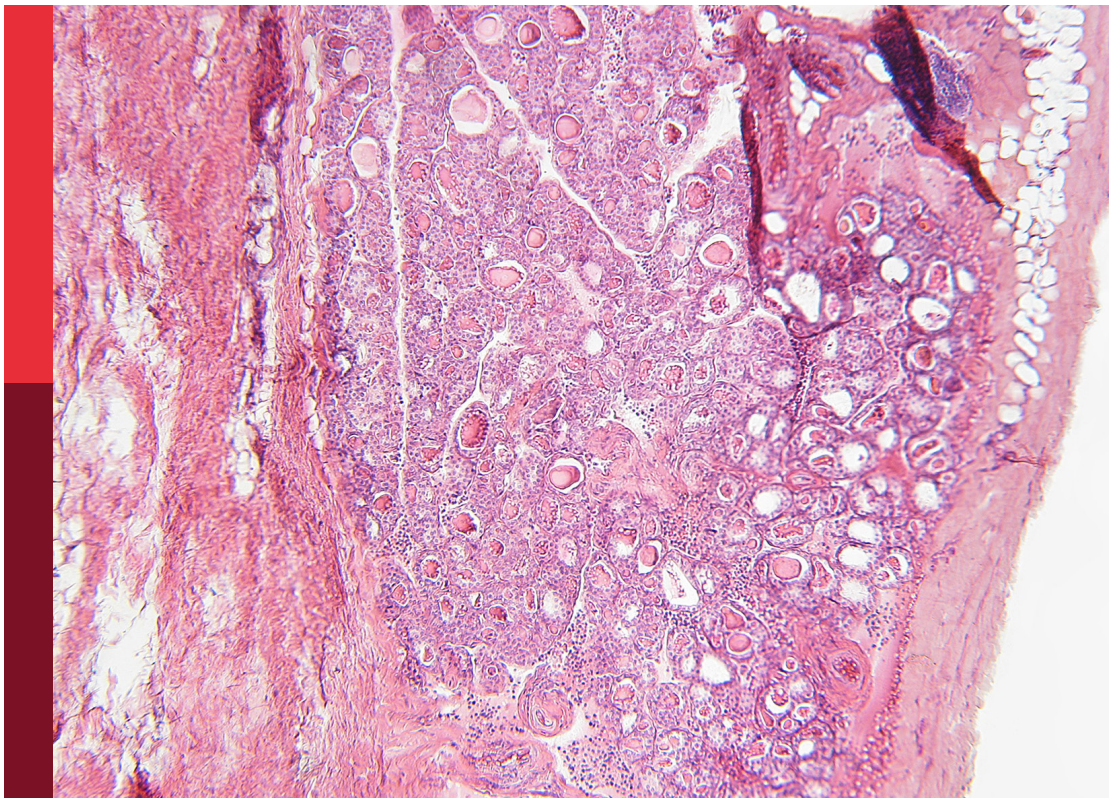

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Functional neuroendocrine tumors

Topic editors
Bojana Popovic — University of Belgrade, Serbia
Aviral Singh — Genesis Cancer Care Australia Pty Ltd, Australia
Francesca Spada — European Institute of Oncology (IEO), Italy

Topic coordinator
Karin Zibar Tomsic — University Hospital Centre Zagreb, Croatia

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Antonino Belfiore,
University of Catania, Italy

*CORRESPONDENCE
Bojana Popovic
popbojana@gmail.com

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Editorial: Functional neuroendocrine tumors

Bojana Popovic[1,2]*, Francesca Spada[3], Aviral Singh[4,5] and Karin Zibar Tomsic[6]

[1]Clinic for Endocrinology, Diabetes and Metabolic Diseases, University Clinical Centre of Serbia, Belgrade, Serbia, [2]Faculty of Medicine, University of Belgrade, Belgrade, Serbia, [3]European Institute of Oncology, IEO, IRCCS, Milan, Italy, [4]Theranostics (Oncology), GenesisCare Australia Pty Ltd, Perth, WA, Australia, [5]Department of Nuclear Medicine, Fiona Stanley Hospital, South Metropolitan Health Service, Perth, WA, Australia, [6]Department of Endocrinology and Diabetology, University Hospital Centre Zagreb, Zagreb, Croatia

KEYWORDS

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Editorial on the Research Topic
Functional neuroendocrine tumors

One of the authentic features of neuroendocrine tumors (NETs) is a potential to synthesize and secrete biogenic peptides and hormones, which can cause recognizable clinical hormone syndromes. These functional NETs are a minority compared to non-functional tumors (i.e. 10-40% in a group of pancreatic NETs), yet they pose significant diagnostic and therapeutic challenges (1). In any case of functional NET, hormonal hypersecretion syndrome leads to excessive morbidity, sometimes more threatening than the malignancy itself (2). Despite the advances in diagnostics and therapy, many issues are still unresolved. This Research Topic aims to address the emerging diagnostic and management strategies, some of which point to the specific biology of these tumors.

At present, the diagnosis of functional NETs relies on clinical presentation and corresponding hormonal secretory profile. Bolduan et al. bring an entirely new perspective by analyzing the expression of sortilin (neurotensin receptor 3), a transmembrane receptor involved in intracellular trafficking of various proteins. Sortilin is associated with several metabolic, neurologic and inflammatory conditions, and has also been shown to contribute to tumorigenesis by promoting cell adhesion and migration (3, 4). Bolduan et al. demonstrate that sortilin expression is twice as high in functional compared to non-functional NETs of different primary origins. This positions sortilin as a potential biomarker for functional NETs, but also as a therapeutic target since the inhibition of sortilin reduced cellular serotonin concentrations in serotonin-producing cell lines.

From a diagnostic and therapeutic standpoint, the expression of somatostatin receptors, particularly type 2 (SSTR2), in various types of NETs is crucial for both localization and treatment with radiolabeled or synthetic somatostatin analogues. The expression of SSTR2 is under negative control of glucocorticoids (5), which makes functional diagnostics and treatment challenging in patients with Cushing's syndrome due to ACTH secreting NET. Pivonello et al. tested the ability of a highly selective non-steroidal GR modulator relacorilant to overcome glucocorticoid induced suppression of SSTR2. In an experimental model of murine At-T20 cell line relacorilant inhibited dexamethasone-mediated reduction of SSTR2A/2B mRNA in a concentration-dependent manner. The authors also demonstrated upregulation of SSTR2

with relacorilant in 4 patients with ACTH-secreting NETs (2 patients with ectopic ACTH secreting NETs (2 patients with ectopic Cushing's syndrome due to ACTH-secreting lung NETs, and 2 patients with Cushing's disease due to ACTH-secreting pituitary adenoma).

Given the high rate of surgical cure, preoperative localization of insulinoma is of utmost importance. However, this can be very challenging as these tumors often measure less than 2 cm, may be multifocal, and are sometimes associated with nesidioblastosis. In cases of diagnostic failure with standard morphological imaging (contrast-enhanced CT, MRI and EUS), functional imaging with ^{68}Ga-DOTA-exendin-4-PET/CT has proven to be the most sensitive method, due to the high expression of GLP-1 receptors in benign insulinomas (6). Since the availability of this method is very limited, and sensitivity of ^{68}Ga-DOTA-SSA or ^{18}F-DOPA PET insufficient, intraarterial calcium stimulation with venous sampling (ASVS) can be utilized as a highly sensitive and specific alternative. Halmi et al. present institutional experience with ASVS in preoperative localization of insulinoma, providing a detailed methodology and interpretation of results in 9 patients. The authors provide a detailed comparison of ASVS with other methods for localization of insulinoma.

As most insulinomas are benign solitary tumors, minimally invasive procedures have emerged as optimal approaches, offering clinical success with fewer adverse events compared to classical open surgery. These include not only minimal invasive surgery (MIS) but also interventional procedures like EUS-guided radiofrequency ablation (RFA) (7). In a meta-analysis, Xiao et al. compared MIS to EUS-guided ablation. Even though the latter had higher recurrence rates, it was associated with lower adverse event rates and shorter hospital stays, making it a viable option for poor surgical candidates.

Since the first report in 2001 (8), only a few other publications presented the use of RFA in the treatment of pheochromocytoma/paraganglioma (PPGL), either for metastatic lesions or small inoperable primary tumors. Magalhaes et al. bring an additional perspective on the utilization of RFA by treating a large, locally aggressive and functional retroperitoneal PGL. Performed by an experienced team, the procedure resulted in a remarkable overall tumor, clinical and biochemical responses. The authors also provide an overview of other potential treatment options for patients who are unsuitable for surgery.

Peptide receptor radionuclide therapy (PRRT) has rarely been examined exclusively in functional NETs. Unlike some anticancer therapies, PRRT has both antiproliferative effect and provides symptom control through reduction in hormonal secretion (9, 10). Nonetheless, certain studies suggest worse overall response rate in functional NETs compared to non-functional tumors (11). In a retrospective study of 51 patients with NETs of various primary locations, Vukomanovic et al. identified tumor functionality as the strongest predictor of poorer survival. By analyzing a large group of 447 pancreatic neuroendocrine neoplasm (pNEN) patients treated with PRRT, Singh et al. developed a clinicopathological and imaging parameter-based nomogram (PANEN-N) for predicting overall survival following PRRT in patients with pNEN. Tumor functionality was shown to be one of the negative predictors of survival.

The final segments of this Research Topic present rare types of functional NETs. Zhao et al. describe an extremely rare case of ectopic ACTH secreting appendiceal NET. By analyzing this patient and 7 other cases reported in the literature so far, the authors thoroughly describe all diagnostic pitfalls regarding this entity.

As much as any case of ectopic Cushing's syndrome can lead to an unnecessary pituitary surgery, this risk is even greater with ectopic acromegaly. Zendran et al. present an extensive review of 127 published cases of ectopic acromegaly (mostly originating from the lung and the pancreas) covering a broad range of clinical characteristics. Since it can be extremely challenging to differentiate pituitary from ectopic acromegaly solely based on clinical presentation, the authors focus on all diagnostic difficulties and present therapeutic options.

Nguyen et al. give a presentation of an extremely rare type of paraganglioma located in the urinary bladder (UBPGL). These rare tumors can present with symptoms and signs of catecholamine excess that are characteristically triggered by micturition. In addition to describing diagnostic approach and surgical treatment options, the authors give a detailed methodology and interpretation of molecular testing for both germline and somatic pathogenic variants that cause paraganglioma.

Lorenço et al. draw attention to insulinomatosis, a rather novel entity that causes persistent hyperinsulinemic hypoglycemia. As in the first formal description in 2009, insulinomatosis represents multiple micro- and macrotumors as well as cell clusters that exclusively produce insulin and arise in the pancreas without genetic background (i.e. syndrome of multiple endocrine neoplasia type 1 – MEN1) (12). The authors present four cases in detail, emphasizing the morphological/immunohistochemical specificities of the disease, and the challenges of achieving surgical cure.

Finally, Cidade-Rodrigues et al. present another rare pancreatic endocrine disorder that only recently reemerged in the literature, which is alpha-cell hyperplasia (ACH). The authors give an informative overview of three types of ACH (reactive, functional and non-functional) and explain differences in their clinical presentation, blood glucagon levels and in the potential to develop into pancreatic NETs. They also present an illustrative case of non-functional ACH with glucagon-producing PNET.

We hope that this Research Topic will be a valuable addendum to current knowledge regarding NETs, and that it will inspire future research.

Author contributions

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Conflict of interest

Author AS was employed by the company GenesisCare Australia Pty Ltd.

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HYPOTHESIS AND THEORY
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Glucocorticoid Receptor Antagonism Upregulates Somatostatin Receptor Subtype 2 Expression in ACTH-Producing Neuroendocrine Tumors: New Insight Based on the Selective Glucocorticoid Receptor Modulator Relacorilant

*Rosario Pivonello[1], Pamela N. Munster[2], Massimo Terzolo[3], Rosario Ferrigno[1], Chiara Simeoli[1], Soraya Puglisi[3], Utsav Bali[4] and Andreas G. Moraitis[5]**

[1] Dipartimento di Medicina Clinica e Chirurgia, Sezione di Endocrinologia, Università Federico II di Napoli, Naples, Italy, [2] Department of Medicine (Hematology/Oncology), University of California San Francisco, San Francisco, CA, United States, [3] Department of Clinical and Biological Sciences, San Luigi Gonzaga Hospital, University of Turin, Orbassano, Italy, [4] Bioscience Department, Sygnature Discovery Ltd, Nottingham, United Kingdom, [5] Drug Research and Development, Corcept Therapeutics, Menlo Park, CA, United States

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Edited by:
Riccarda Granata,
University of Turin, Italy

Reviewed by:
Francesco Cavagnini,
Istituto Auxologico Italiano (IRCCS), Italy
Monica Marazuela,
Autonomous University of Madrid, Spain

***Correspondence:**
Andreas G. Moraitis
amoraitis@corcept.com

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Somatostatin exhibits an inhibitory effect on pituitary hormone secretion, including inhibition of growth hormone and adrenocorticotropic hormone (ACTH), and it can have antisecretory and antitumor effects on neuroendocrine tumors (NETs) that express somatostatin receptors. Although the precise mechanism remains unclear, the finding that glucocorticoids downregulate somatostatin receptor subtype 2 (SSTR2) expression has been used to explain the lack of efficacy of traditional SSTR2-targeting analogs in patients with ACTH-secreting NETs. Glucocorticoid receptor (GR) antagonism with mifepristone has been shown to reverse the glucocorticoid-induced downregulation of SSTR2; however, the effects of GR modulation on SSTR2 expression in ACTH-secreting NETs, particularly corticotroph pituitary tumors, are not well known. The current study presents new insight from *in vitro* data using the highly selective GR modulator relacorilant, showing that GR modulation can overcome dexamethasone-induced suppression of SSTR2 in the murine At-T20 cell line. Additional data presented from clinical case observations in patients with ACTH-secreting NETs suggest that upregulation of SSTR2 *via* GR modulation may re-sensitize tumors to endogenous somatostatin and/or somatostatin analogs. Clinical, laboratory, and imaging findings from 4 patients [2 ACTH-secreting bronchial tumors and 2 ACTH-secreting pituitary tumors (Cushing disease)] who were treated with relacorilant as part of two clinical studies (NCT02804750 and NCT02762981) are described. In the patients with ectopic ACTH secretion, SSTR2-based imaging (Octreoscan and ^{68}Ga-DOTATATE positron emission tomography) performed before and after treatment with relacorilant showed increased radiotracer uptake by the tumor following treatment with relacorilant without change in tumor size at

computed tomography. In the patients with Cushing disease who received relacorilant prior to scheduled pituitary surgery, magnetic resonance imaging after a 3-month course of relacorilant showed a reduction in tumor size. Based on these findings, we propose that GR modulation in patients with ACTH-secreting NETs upregulates previously suppressed SSTR2s, resulting in tumor-specific antisecretory and anti-proliferative effects. The effect of relacorilant on pituitary corticotroph tumors is being investigated in an ongoing phase 3 study (NCT03697109; EudraCT 2018-003096-35).

Keywords: glucocorticoid, cortisol, somatostatin, relacorilant, neuroendocrine tumor, adrenocorticotropic hormone, Cushing disease, ectopic ACTH syndrome

INTRODUCTION

Somatostatin receptors (SSTRs) are expressed in organs and tissues throughout the body (1–3) as well as in many different tumor types, including neuroendocrine tumors (NETs) (4–7)—a heterogeneous group of neoplasms (eg, pituitary tumors, carcinoid tumors, gastroenteropancreatic tumors, phaeochromocytomas, medullary thyroid carcinomas, and small cell tumors of the lung and prostate) arising from neuroendocrine cells. SSTRs include five different subtypes (SSTR1-5) belonging to the G-protein-coupled receptor class (1, 7). SSTR2 and SSTR5 are predominately expressed in endocrine tissues (eg, pituitary gland), and SSTR2 is one of the most abundantly expressed receptor subtypes in many NETs (2, 5, 6, 8). The presence of SSTR2 on NETs has led to the use of synthetic somatostatin analogs (eg, octreotide and lanreotide) (9–13) and radiolabeled somatostatin analogs (eg, ^{111}In-pentetreotide [Octreoscan] and ^{177}Lu-DOTATATE) (14, 15) for tumor localization and treatment.

Somatostatin is a potent inhibitor of pituitary hormone secretion, including inhibition of growth hormone and adrenocorticotropic hormone (ACTH) secretion (1, 8, 16). Similarly, somatostatin analogs have antisecretory effects and show antitumoral activity (9–12). However, many patients with NETs develop resistance or do not respond to somatostatin analogs targeting SSTR2. For instance, octreotide is partially effective in patients with extra-pituitary corticotroph tumors responsible for ectopic Cushing syndrome or ectopic ACTH secretion and is generally ineffective in patients with pituitary corticotroph tumors responsible for the most common form of ACTH-dependent Cushing syndrome, namely, Cushing disease (17, 18). Pituitary corticotroph tumors have lower expression of SSTR2 and higher expression of SSTR5 and dopamine type 2 (D2) receptors (19, 20), leading to the clinical use of dopamine agonist cabergoline, which has high affinity for D2, and the multi-somatostatin analog pasireotide, which has high affinity for SSTR5 (21, 22), over analogs targeting SSTR2.

Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; CT, computed tomography; DMEM, Dulbecco's minimal essential medium; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GR, glucocorticoid receptor; LAR, long-acting release; MRI, magnetic resonance imaging; NET, neuroendocrine tumor; PET, positron emission tomography; qPCR, quantitative PCR; SD, standard deviation; SGRM, selective glucocorticoid receptor modulator; SSTR, somatostatin receptor; SSTR1-5, somatostatin receptor subtypes 1-5.

The lack of efficacy of somatostatin analogs targeting SSTR2 in patients with Cushing disease supports the hypothesis that SSTR2 is downregulated by glucocorticoids. *In vitro* studies have shown that dexamethasone treatment of At-T20 corticotroph tumor cells induced significant suppression of SSTR2 messenger ribonucleic acid (mRNA) expression (23). Glucocorticoids have also been shown to attenuate the inhibitory effects of octreotide on ACTH release *in vitro* (18, 23). This downregulation by glucocorticoids might explain not only the lack of efficacy of SSTR2-targeting somatostatin analogs in patients with Cushing disease but also their partial effect in patients with ectopic ACTH syndrome. The ACTH-secretory capacity of these latter patients' tumors has been shown to be more "resistant" to the negative feedback of cortisol excess, as an intact glucocorticoid signaling pathway is not always present within these cells (24–27).

The glucocorticoid receptor (GR) antagonist mifepristone, a non-selective steroidal GR antagonist with progesterone receptor activity, has been shown to reverse the inhibitory effects of glucocorticoids on SSTR2 mRNA expression in the human neuroendocrine cell lines BON (carcinoid) and TT (medullary thyroid carcinoma) (27). Treatment with dexamethasone resulted in 71% and 69% reductions in SSTR2 mRNA expression in BON and TT cells, respectively. Co-administration with mifepristone completely inhibited the dexamethasone-mediated downregulation of SSTR2 mRNA. The *in vitro* effects of mifepristone on SSTR2 expression in corticotroph tumor cell lines have not been studied. A clinical report of two patients with ectopic ACTH syndrome treated with mifepristone noted upregulation of SSTR2 on diagnostic imaging (24). However, mifepristone treatment in patients with ACTH-dependent Cushing syndrome, including ectopic ACTH syndrome, has not been shown to affect tumor growth or decrease the ACTH-secretory capacity of the tumor (28, 29).

Relacorilant (CORT125134, Corcept Therapeutics, Menlo Park, CA) is a highly selective non-steroidal GR modulator (SGRM) that modulates cortisol activity (30) and, unlike mifepristone, lacks progesterone receptor activity. Relacorilant is under clinical investigation for the treatment of patients with endogenous Cushing syndrome (NCT03697109, NCT04308590, and NCT04373265). The effects of relacorilant on SSTR2 expression have not been previously assessed, and the diagnostic and therapeutic implications of the relationship between GR modulation and SSTR2 in patients with ACTH-secreting NETs remain unclear.

HYPOTHESIS

This report provides new insight into the relationship between GR modulation and SSTR2, suggesting that GR modulation with relacorilant may overcome the glucocorticoid-induced suppression of SSTR2, enabling SSTR2-mediated effects in ACTH-secreting NETs (**Figure 1**). Presented in this report are *in vitro* laboratory data and findings from several clinical observations supporting the hypothesis that SGRM-induced upregulation of SSTR2 by relacorilant may enhance tumor localization *via* SSTR imaging and may also sensitize tumors to the antitumor effects of somatostatin and its analogs.

EXPERIMENTAL STUDY: EFFECT OF RELACORILANT ON SSTR2A/2B mRNA EXPRESSION

An *in vitro* analysis was undertaken to assess, for the first time, the effects of relacorilant on SSTR2 mRNA in a mouse pituitary corticotroph cell line. In mice, two isoforms of SSTR2, SSTR2A and SSTR2B, have been identified, with human tissues expressing SSTR2A almost exclusively (31, 32). Using the murine At-T20 cell line, a well-studied corticotroph model (23), an assessment of the effects of dexamethasone alone on SSTR2A/2B mRNA levels was conducted followed by an assessment of the effects of dexamethasone and relacorilant combined.

At-T20 cells were cultured in complete medium, and mRNA expression levels of SSTR2A and SSTR2B were determined upon pretreatment for 24 hours across a dexamethasone concentration gradient (detailed Methods can be found at the end of the report). Dexamethasone treatment (100 nM) resulted in a 3-fold and 2.4-fold reduction in SSTR2A and SSTR2B mRNA levels, respectively.

Relacorilant inhibited the dexamethasone-mediated reduction of SSTR2A/2B mRNA in a concentration-dependent manner (**Figure 2**). At relacorilant concentrations greater than 1 μM, an increase in SSTR2A/2B mRNA levels above basal (untreated) levels was observed, reaching an ~1.5-fold increase at 10 μM, the highest relacorilant concentration tested.

CLINICAL OBSERVATIONS

Four patients with NETs received investigational relacorilant as part of two clinical studies: a phase 2 Cushing syndrome study of relacorilant (NCT02804750, EudraCT 2016-000899-23) and a phase 1 oncology study of relacorilant + nab-paclitaxel (NCT02762981). Unique changes to the patients' tumor characteristics were observed, based on magnetic resonance imaging (MRI) or SSTR imaging. In the Cushing syndrome study, radiologic imaging was included as standard of care, outside the study.

Case 1: Effect of Relacorilant on Octreotide Scintigraphy in a Patient With Ectopic Cushing Syndrome

A 46-year-old woman with an ectopic ACTH-secreting tumor was enrolled in the phase 2 Cushing syndrome study of relacorilant and received relacorilant 250 mg/day titrated to 400 mg/day. Octreotide scintigraphy (Octreoscan) performed before relacorilant treatment was positive for a lung lesion, but a CT scan and MRI of the lungs did not show any discrete mass at study entry.

Compared to baseline, repeat octreotide imaging performed after 16 weeks of treatment with relacorilant showed increased uptake at the tumor site (**Figure 3A**). The tumor continued to remain undetectable on repeat CT scans. ACTH and cortisol levels were 66.8 pg/mL (normal range, 6.0–50 pg/mL) and 22.7 μg/dL (normal range, 4.6–20.6 μg/dL), respectively, at baseline. After an initial increase, levels of ACTH and cortisol decreased near or below baseline levels at week 16 (**Figure 3B**), in contrast to the increase seen in patients with ACTH-dependent Cushing syndrome treated with mifepristone (28, 29).

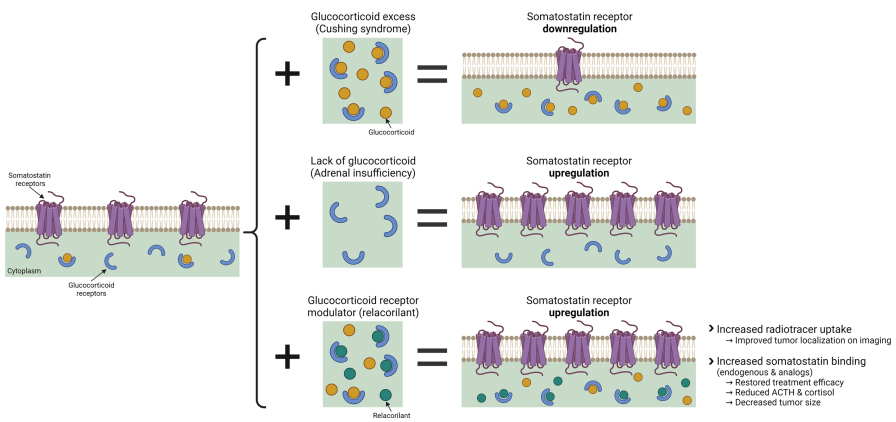

FIGURE 1 | The effects of glucocorticoid and glucocorticoid modulation with relacorilant on SSTR regulation. Created with BioRender.com.

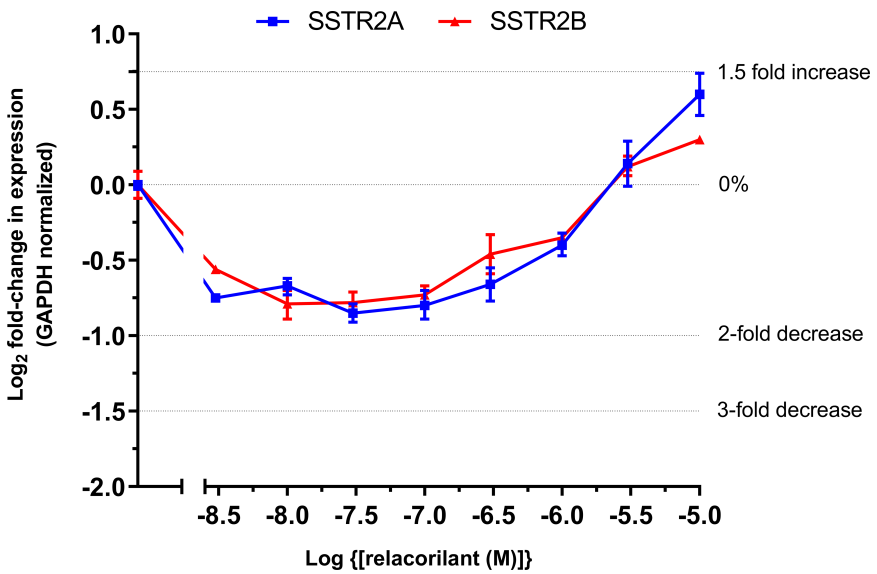

FIGURE 2 | Log_2 fold change in SSTR2 mRNA in murine At-T20 cells upon treatment with increasing concentrations of relacorilant for 24 h in the presence of 100 nM dexamethasone. 0%, 2-fold, and 3-fold inhibition and 1.5-fold increase in levels are highlighted by dotted lines on the y-axis. Zero relative expression is in the absence of dexamethasone. Data points show average fold change compared to baseline and SD error bars. Data are technical replicates with an underlying n=1.

Case 2: Effect of Relacorilant on SSTR Positron Emission Tomography Imaging in a Patient With an ACTH-Secreting Metastatic Bronchial Carcinoid NET

A 68-year-old man with a metastatic carcinoid NET (bronchial primary) was enrolled in the phase 1 oncology study of relacorilant + nab-paclitaxel. The primary tumor pathology was consistent with a typical carcinoid. Previous treatments included octreotide long-acting release (LAR), everolimus, carboplatin + etoposide, sunitinib, and capecitabine + temozolomide. The patient received relacorilant 200 mg/day on the day before, the day of, and the day after nab-paclitaxel infusion (80 mg/m^2 administered on days 1, 8, and 15 of a 28-day cycle). During the study, the patient received octreotide LAR 20 mg monthly.

ACTH [56.4 pg/mL [normal range, 6–50 pg/mL)] and cortisol levels [23.5 μg/dL (normal range, 4.6–20.6 μg/dL)] were elevated in this patient at study baseline (**Figure 4A**). ^{68}Ga-DOTATATE scans with CT showed increased uptake of the radiotracer at lung and bone lesions during relacorilant treatment compared to baseline without an increase in tumor size (**Figures 4B–D**). ^{68}Ga-DOTATATE imaging of the pituitary showed no uptake at baseline (**Figure 4E**). Normally, the pituitary gland expresses SSTR2, and physiologically increased uptake is seen in eucortisolemic patients' DOTATATE scans (33, 34). During relacorilant treatment, however, the pituitary uptake was restored (**Figure 4E**). ACTH and serum cortisol decreased during concomitant relacorilant and octreotide LAR treatment (**Figure 4A**), suggestive of an increased effect of octreotide LAR due to upregulation of SSTR2 without tumor shrinkage.

Cases 3 and 4: Effect of Relacorilant on Pituitary Tumor Size in Two Patients With Cushing Disease

Two patients with *de novo* Cushing disease due to pituitary macroadenomas received relacorilant 100 mg/day titrated to 200 mg/day as part of a phase 2 Cushing syndrome study prior to previously scheduled transsphenoidal pituitary surgery (35). Patient 3 was a 50-year-old woman with a pituitary macroadenoma measuring 10.01 × 6.29 × 4.91 mm on MRI (tumor volume 155 mm^3). Patient 4 was a 43-year-old man with a pituitary macroadenoma measuring 22 × 25 × 26 mm (tumor volume 7,150 mm^3) with suprasellar extension, right displacement of the pituitary stalk, and invasion of the left cavernous sinus on MRI. MRI with gadolinium was conducted before the initiation of relacorilant and within 12 weeks after the last dose of relacorilant. In both patients, imaging revealed reduction in the size of their tumors (**Figures 5A**, **6A**) after treatment with relacorilant. Tumor volume decreased from 155 mm^3 to 84 mm^3 for Patient 3 and from 7,150 mm^3 to 4,389 mm^3 for Patient 4. Changes in ACTH and serum cortisol levels, as shown in **Figures 5B**, **6B**, showed a similar trend to those of Patient 1 (initial increase followed by reduction below pretreatment levels).

DISCUSSION

SSTR2s are expressed in a variety of tumor types, which has led to the use of SSTR2-targeting analogs for diagnosis and treatment. However, the effects of GR modulation on SSTR2

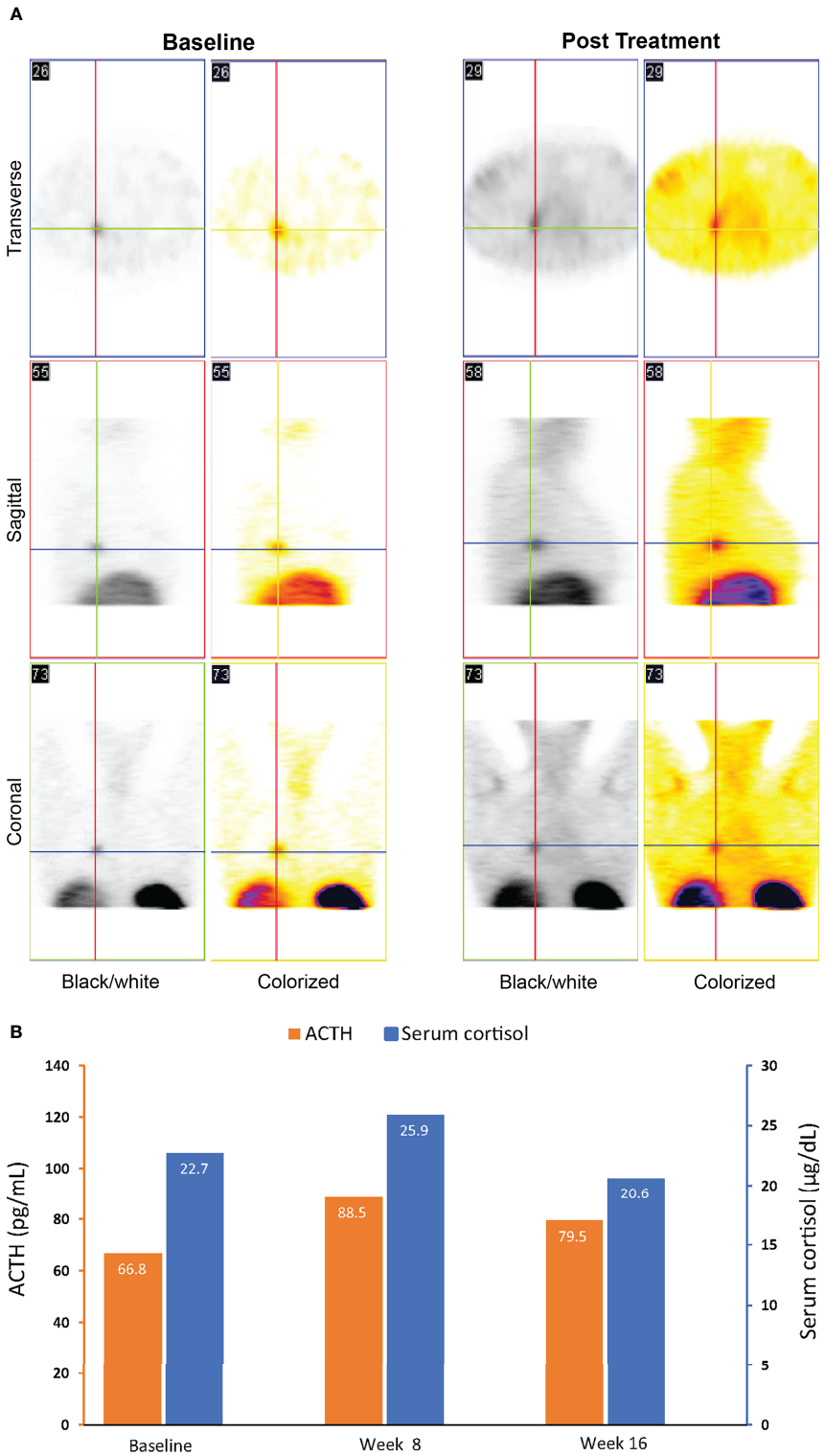

FIGURE 3 | Imaging **(A)** and ACTH and cortisol levels **(B)** for case 1: a 46-year-old woman with an ectopic ACTH-secreting tumor (ectopic Cushing syndrome) treated with relacorilant for 16 weeks. **(A)** Octreotide scintigraphy. Increased uptake on post treatment imaging was consistent with increased expression of SSTR2s following treatment with relacorilant. **(B)** ACTH and cortisol levels before (baseline) and during relacorilant treatment. Normal laboratory ranges: ACTH, 6.0-50 pg/mL; serum cortisol, 4.6-20.6 µg/dL. To convert ACTH values from pg/mL to pmol/L, multiply by 0.22. To convert serum cortisol from µg/dL to nmol/L, multiply by 27.6. ACTH, adrenocorticotropic hormone.

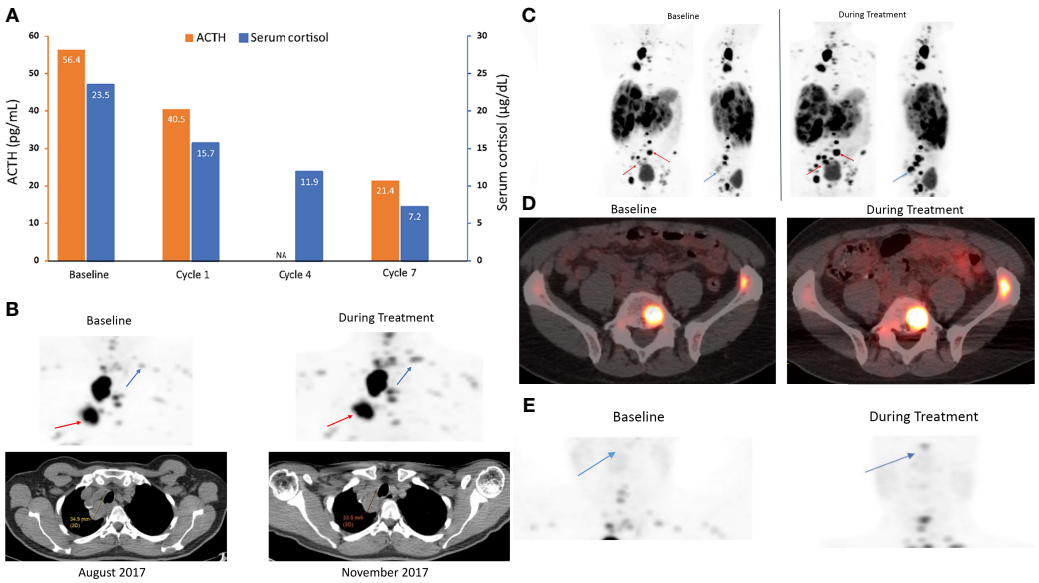

FIGURE 4 | ACTH and serum cortisol levels **(A)** and ^{68}Ga-DOTATATE scans **(B–E)** in case 2: a 68-year-old man with a metastatic carcinoid NET treated with 7 cycles of relacorilant + nab-paclitaxel. **(A)** ACTH and cortisol levels before (baseline) and during relacorilant + nab-paclitaxel treatment. Patient received concomitant somatostatin analog. Normal laboratory ranges: ACTH, 6-50 pg/mL; morning serum cortisol, 4.6-20.6 µg/dL. To convert ACTH values from pg/mL to pmol/L, multiply by 0.22. To convert serum cortisol from µg/dL to nmol/L, multiply by 27.6. **(B)** ^{68}Ga-DOTATATE scan showed multiple lung and bone lesions at baseline before treatment with relacorilant. Repeat scan during treatment with relacorilant showed increased uptake without change in size of the lesions on CT. **(C)** ^{68}Ga DOTATATE scan showed multiple lung, liver, and bone lesions at baseline before treatment with relacorilant. Repeat scan during treatment with relacorilant showed increased uptake. **(D)** ^{68}Ga DOTATATE scan of L5 and left iliac bone lesions at baseline. Repeat scan during treatment with relacorilant showed increased uptake. **(E)** Compared with the ^{68}Ga-DOTATATE scan before treatment with relacorilant, the repeat scan during relacorilant treatment showed increased uptake at the pituitary gland. ACTH, adrenocorticotropic hormone; NA, not available.

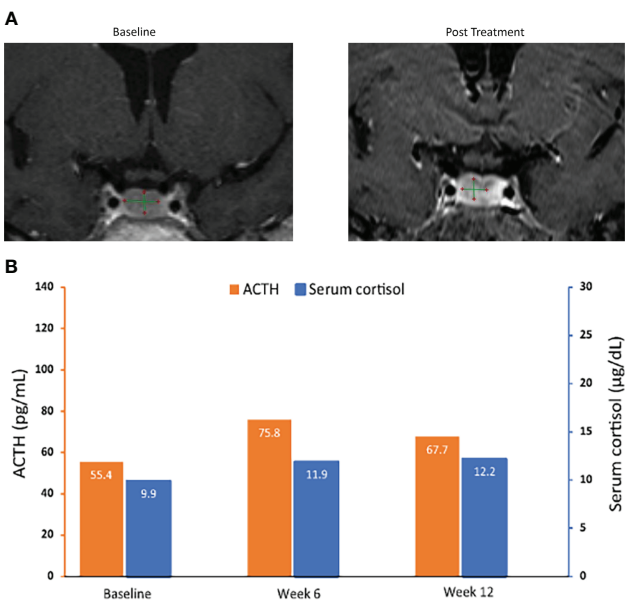

FIGURE 5 | MRI of pituitary macroadenomas **(A)** and ACTH and cortisol levels **(B)** in case 3: a 50-year-old woman with Cushing disease treated with relacorilant for 12 weeks. **(A)** Coronal post contrast T1-weighted MRI obtained at diagnosis (left image) after administration of gadolinium showed a nodular lesion with reduced enhancement in the median and paramedian anterior part of the sellar region compatible with pituitary macroadenoma. It measured 10.01 × 6.29 × 4.91 mm. Pituitary MRI obtained (right image) within 12 weeks after the last dose of relacorilant showed a reduction in the size of the macroadenoma (8.04 × 5.70 × 3.65 mm). **(B)** ACTH and cortisol levels. Normal laboratory ranges: ACTH, 6.0-50 pg/mL; serum cortisol, 4.6-20.6 µg/dL. To convert ACTH values from pg/mL to pmol/L, multiply by 0.22. To convert serum cortisol from µg/dL to nmol/L, multiply by 27.6. ACTH, adrenocorticotropic hormone.

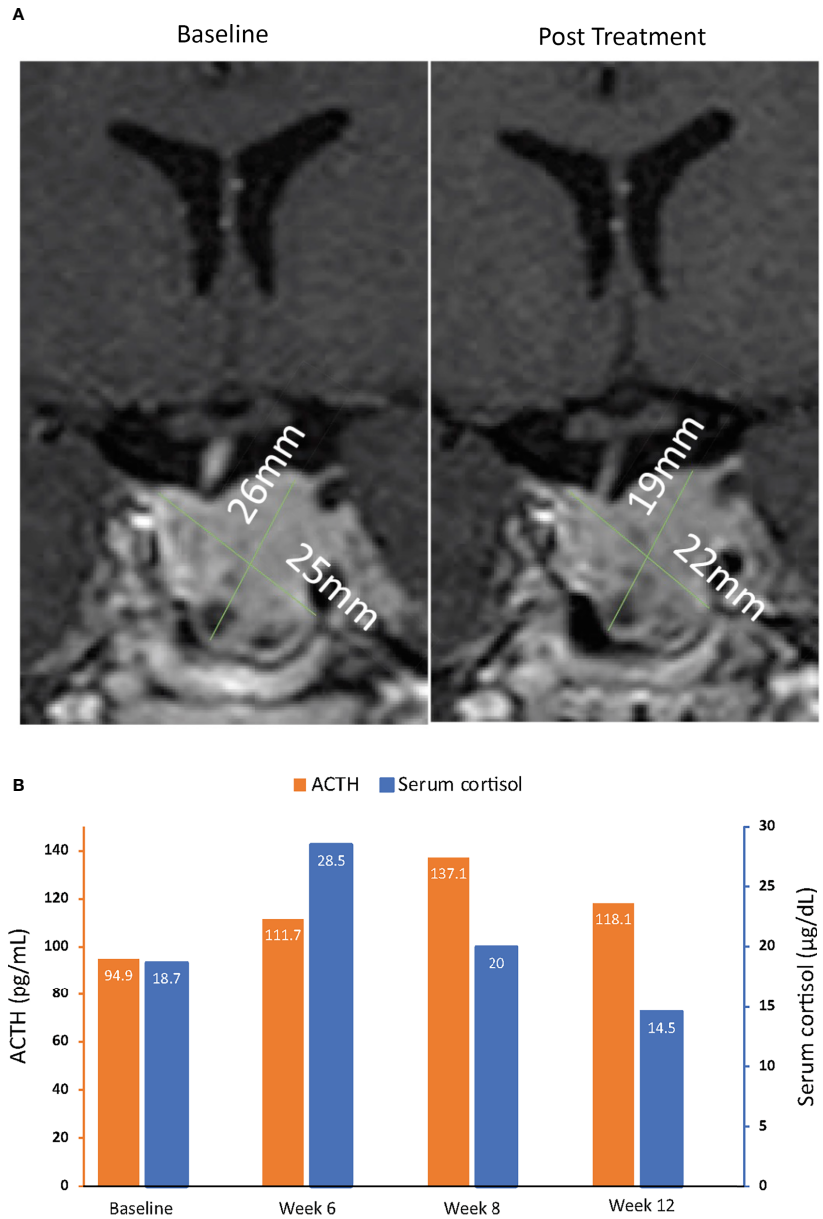

FIGURE 6 | MRI of pituitary macroadenomas **(A)** and ACTH and cortisol levels **(B)** in case 4: a 43-year-old man with Cushing disease treated with relacorilant for 12 weeks. **(A)** Coronal post contrast T1-weighted MRI obtained at diagnosis (left image) after administration of gadolinium showed a pituitary macroadenoma measuring 22 × 25 × 26 mm with suprasellar extension, right displacement of the pituitary stalk, and invasion of the left cavernous sinus. The tumor was isointense to the gray matter and slightly inhomogeneous for the presence of cystic changes in its lower aspect. MRI of the hypophysis obtained within 12 weeks after the last dose of relacorilant (right image), after treatment with relacorilant, showed a reduction in the size of the macroadenoma (21 × 22 × 19 mm). **(B)** ACTH and cortisol levels. Normal laboratory ranges: ACTH, 6.0-50 pg/mL; serum cortisol, 4.6-20.6 µg/dL. To convert ACTH values from pg/mL to pmol/L, multiply by 0.22. To convert serum cortisol from µg/dL to nmol/L, multiply by 27.6. ACTH, adrenocorticotropic hormone.

have not been well studied. The preclinical data as well as several clinical case observations reported here illustrate the potential effects of relacorilant on ACTH-secreting NETs. In the *in vitro* analysis, selective GR modulation with relacorilant inhibited glucocorticoid-mediated suppression of SSTR2 in the murine At-T20 cell line. Imaging and laboratory data from four patients with ACTH-secreting NETs showed increased uptake of radiotracer *via* SSTR2-based imaging and a reduction in pituitary corticotroph tumor size following treatment with relacorilant.

Glucocorticoids induce downregulation of SSTR2, which can explain the low SSTR2 expression reported in tumors derived from patients with Cushing disease (20). In the At-T20 cell line, dexamethasone-mediated suppression of SSTR2 mRNA was

reversed by selective GR modulation with relacorilant. At higher concentrations of relacorilant, SSTR2 mRNA expression even increased above basal levels in the At-T20 cell line. Studies of the regulatory effects of somatostatin in normal rat pituitary cells and in healthy humans have shown that treatment with somatostatin does not inhibit basal or CRH-stimulated ACTH secretion (1, 16, 36). However, in patients with Nelson's syndrome and elevated plasma ACTH following bilateral adrenalectomy for Cushing disease, somatostatin infusion was shown to decrease ACTH secretion (37). In a separate analysis of patients with primary adrenal insufficiency, somatostatin injection also resulted in a reduction in ACTH (38). Patients in both studies had been receiving glucocorticoid replacement therapy, which was withheld prior to the administration of somatostatin. Together with the findings of the current study, these data suggest that either a lack or an excess of glucocorticoids may lead to abnormal SSTR2 expression (upregulation in adrenal insufficiency and downregulation in Cushing syndrome). GR modulation with relacorilant may overcome the inhibitory effect of glucocorticoids on SSTR2 expression, restoring the efficacy of the endogenous somatostatin and exogenous somatostatin analogs.

The two patients with ectopic tumors showed increased uptake of the radioactive somatostatin analog used for imaging NETs with relacorilant administration. This result is notable, as in up to 27% of patients with ectopic Cushing syndrome, the tumor source is not localized even after long-term follow-up (15). While increased uptake could also have occurred because of interval increases in the size of the lesions, there was no evidence of a change in tumor size in these patient cases based on CT imaging. SSTR2 is normally expressed in the pituitary gland (33, 34), and increased uptake on ^{68}Ga-DOTATATE scan is seen in eucortisolemic patients. In the patient with an ACTH-secreting metastatic bronchial NET, there was no physiologic uptake of ^{68}Ga-labeled somatostatin analog in the pituitary gland at baseline. Of note, ectopic ACTH secretion is common in lung carcinoid tumors but is not always associated with overt cortisol excess (39). Because of the low differentiation of these tumors, the ACTH secreted by the tumors in most cases is biologically inactive (ACTH-like peptides, also referred to as ACTH precursors) but can cross-react with commercially available ACTH assays. The lack of ^{68}Ga-labeled somatostatin analog uptake in the pituitary gland, along with the elevated serum cortisol and ACTH levels at baseline, suggests that this patient had some degree of cortisol excess at baseline; however, no formal evaluation for Cushing syndrome (eg, urinary free cortisol, dexamethasone suppression testing, or late-night salivary cortisol) was required for enrollment in the oncology study. In this patient, treatment with relacorilant reversed the effect of cortisol on the SSTR2s in the pituitary, resulting in restoration of SSTR2 expression in the pituitary and visualization in the repeat scans. This patient was also receiving concomitant nab-paclitaxel, but the authors are not aware of any studies suggesting that nab-paclitaxel has an effect on SSTR2. These cases highlight the potential effects of GR modulation with relacorilant in instances of ectopic ACTH secretion and suggest that relacorilant can enhance SSTR-based imaging, which may improve diagnostic accuracy.

The increased expression of SSTR2 with relacorilant was also supported by changes in the patients' ACTH and cortisol levels. In patients 1, 3 and 4, ACTH and cortisol levels initially increased and then decreased later during treatment. The initial increase was expected based on experience with the GR antagonist mifepristone (28). The mechanisms for the eventual decrease in ACTH and cortisol levels with relacorilant are not yet fully understood. While the decrease in ACTH and cortisol during relacorilant treatment might reflect an exhaustion of the stimulatory effect of relacorilant, this effect has not been observed with mifepristone. In patients with Cushing disease, mifepristone use is associated with dose-dependent increases in ACTH and cortisol, and ACTH levels generally remain elevated over time with continued treatment (29). Furthermore, mifepristone pretreatment in patients with Cushing disease was not shown to affect ACTH and cortisol levels in response to acute octreotide administration (40). The overexpression of SSTR2 observed at higher doses of relacorilant in the *in vitro* analysis offers another possible explanation for the effects observed with relacorilant. In the previous studies of mifepristone in NET cell lines, mifepristone reversed the effects of dexamethasone but was not associated with SSTR2 overexpression (27). Together, these findings suggest a potential difference in effect between mifepristone and relacorilant, in which relacorilant-induced increased SSTR2 expression on the tumor can increase the efficacy of endogenous and exogenous somatostatin on ACTH secretion and tumor proliferation. In Patient 2, who received octreotide LAR along with relacorilant, the levels of ACTH and cortisol decreased throughout relacorilant treatment, without the initial increase that was observed in patients not receiving concurrent somatostatin analogs. A possible explanation is that in the presence of high somatostatin levels achieved with exogenous administered somatostatin analogs, even lower doses of relacorilant may lead to sufficient upregulation of SSTR2 to enhance the effect of somatostatin analogs on the secretory function of the NET; however, this would need to be confirmed by further research in a larger, more homogenous population.

Somatostatin analogs have been shown to inhibit tumor hypersecretion of peptides and slow tumor growth in gastrointestinal cancers, including NETs (9–12), and expression of SSTR2 has been associated with improved survival in patients with gastropancreatic NETs (41). The antisecretory and anti-proliferative effects of somatostatin and its analogs are mediated by both direct and indirect mechanisms (3, 42). Direct effects include cell cycle arrest, inhibition of growth factor signaling, and apoptosis through the regulation of MAP kinase and phosphotyrosine phosphatase activities upon activation of SSTR2. Indirect effects include inhibition of tumor angiogenesis, secretion of tumor-promoting signals from immune cells, and secretion of growth factor. Octreotide has also been shown to reduce tumor volume in patients with growth hormone-secreting and thyroid-stimulating hormone-secreting pituitary tumors (3, 42). In the clinical case examples of the

current study, we observed a decrease in tumor size in two patients with *de novo* Cushing disease due to macroadenomas (patients 3 and 4) following treatment with relacorilant. While spontaneous tumor regression cannot be ruled out (although extremely rare) (43), the changes in ACTH and cortisol levels that occurred in these patients during relacorilant treatment, characterized by early increases followed by reductions later during treatment, are consistent with the hypothesized inhibition of ACTH by endogenous somatostatin due to upregulation of SSTR2, as also seen in Patient 1. Together, these data suggest that relacorilant-mediated upregulation of SSTR2 provides more targets for somatostatin and somatostatin analogs, which can lead to tumor shrinkage in ACTH-secreting pituitary tumors.

There are a number of limitations to the data presented in this report. Although the murine corticotroph tumor At-T20 cell line is the most frequently studied model for Cushing disease, the *in vitro* findings may not necessarily translate to human cells. Thus, one cannot rule out the possibility of another mechanism for the trends in ACTH and cortisol levels observed in the patient cases. The small number and heterogeneous nature of the clinical cases, including concomitant therapies, must also be considered when interpreting the clinical observations. The lack of SSTR2 imaging or immunohistochemical analysis of SSTR2 expression before and after relacorilant treatment is another limitation for patient cases 3 and 4; however, SSTR2 imaging is not part of the standard diagnostic evaluation of ACTH-secreting pituitary tumors (44).

Based on these findings, additional examination should be carried out to formally assess and elucidate the tumor-specific effects of relacorilant in patients with ACTH-producing NETs to determine whether it has a potential diagnostic role and antitumor effects. A therapeutic trial that could sensitize ACTH-secreting pituitary tumors to endogenous somatostatin prior to surgery could be beneficial, particularly in patients with invasive macroadenomas. Ongoing preclinical studies in human pituitary cell lines and the phase 3 study of relacorilant in patients with Cushing syndrome (clinicaltrials.gov NCT03697109), which includes tumor imaging, may provide additional insight.

IN VITRO METHODS

Cell Culture

At-T20 mouse pituitary tumor cells were obtained from ATCC (CCL-89) and cultured in high-glucose Dulbecco's minimal essential medium (DMEM) complete media [10% fetal calf serum + penicillin and streptomycin (Penn/Strep)]. For compound treatment, 96-well plates were seeded with 50,000 cells and allowed to adhere for 6 h in complete media containing charcoal-stripped serum. Agonist treatment with a dexamethasone concentration gradient was carried out for 24 h [0.2% final dimethylsulfoxide (DMSO)]. For antagonist assays, the cells were pre-treated for 30 min with a relacorilant concentration gradient prior to the addition of 100 nM (EC_{max}) dexamethasone and incubated for 24 h (0.2% final DMSO). After treatment, the medium was removed, and cells were lysed directly in lysis buffer (buffer RLT Qiagen RNeasy) followed by total RNA extraction.

RNA Isolation

Total RNA from At-T20 cellular lysates was isolated using Qiagen RNeasy Mini kit (Qiagen 74104) by following the manufacturer's recommended instructions. RNA was eluted in a 100 µL volume of nuclease-free elution buffer and stored at -20°C until use. Contaminating genomic DNA was eliminated by the inclusion of a deoxyribonuclease treatment step (deoxyribonuclease I at 8 U per 100 µL of eluate) (45). Total RNA yield and purity were measured by spectrophotometric analysis (A_{260} to A_{280} ratio) using a Nanodrop 1000 instrument.

Reverse Transcription and Real-Time Quantitative PCR

Reverse transcription and real-time qPCR were performed as previously described (45). Total RNA (0.2–1.0 µg) was reverse transcribed in 20 µL reaction volume using a high-capacity cDNA reverse transcription kit (Applied Biosystems; 4368814) with random hexamers. Real-time qPCR experiments were performed in a 96-well plate using an Applied Biosystems StepOnePlus real-time PCR instrument. For each sample, the expression of SSTR2 was compared with the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, with the latter included as a housekeeping gene for endogenous control. Taq-Man gene expression assays were obtained from Life Technologies and consisted of a 20X mix of unlabeled PCR primers for mouse SSTR2A (Life Technologies; Mm03015782_s1) and mouse SSTR2B (Life Technologies; Mm00436685_g1) and for mouse GAPDH (Life Technologies; Mm99999915_g1) and TaqMan MGB probe (FAM dye labelled). The reaction mixture for real-time qPCR contained 9.0 µL cDNA solutions (20–100 ng). Each of the two primers and the MGB probe were used at 0.9 µM and 0.25 µM, respectively, and 1X TaqMan Universal Master Mix II with UNG (Applied Biosystems; 4440038). The mixture was activated (2 min, 50°C), denatured (10 min, 95°C) and subjected to 40 amplification cycles (15 sec, 95°C; 1 min, 60°C) with a single measurement of fluorescence for both SSTR and GAPDH primer sets.

Data Analysis

TaqMan qPCR data were analyzed using StepOnePlus software version 2.3. Amplification plots were visualized across the entire 96-well plate for SSTR2A/B probe sets and GAPDH. Fractional cycle (C_T) values were returned by manually setting the threshold to intersect at the linear phase of amplification plots (defined manually at 0.1) (45). No-treatment control sample was selected as the calibrator, and the data were analyzed relative to the calibrator. The comparative $\Delta\Delta C_T$ method was used for data analyses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board at each study center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concept and design: AM. Study investigators who provided study materials and/or patients: RP, PM, MT, RF, SP, and CS. Analyzed and interpreted preclinical data: UB and AM. Analyzed and interpreted clinical data: RP, PM, MT, RF, SP, CS, and AM. Wrote manuscript or critically revised it for content: All authors. Reviewed final manuscript and gave approval for submission: All authors.

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CASE REPORT
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Appendiceal Neuroendocrine Tumor Is a Rare Cause of Ectopic Adrenocorticotropic Hormone Syndrome With Cyclic Hypercortisolism: A Case Report and Literature Review

*Yu Xing Zhao[1], Wan Lu Ma[1], Yan Jiang[1], Guan Nan Zhang[2], Lin Jie Wang[1], Feng Ying Gong[1], Hui Juan Zhu[1] and Lin Lu[1]**

[1] Key Laboratory of Endocrinology of National Health Commission, Department of Endocrinology, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China, [2] Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China

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***Correspondence:**
Lin Lu
lulin88@sina.com

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Objective: Ectopic adrenocorticotropic hormone (ACTH) syndrome (EAS) is a condition of hypercortisolism caused by non-pituitary tumors secreting ACTH. Appendiceal neuroendocrine tumor as a rare cause of ectopic ACTH syndrome was reported scarcely. We aimed to report a patient diagnosed with EAS caused by an appendiceal neuroendocrine tumor and summarized characteristics of these similar cases reported before.

Case Report and Literature Review: We reported a case with Cushing's syndrome who was misdiagnosed as pituitary ACTH adenoma at first and accepted sella exploration. Serum and urinary cortisol decreased, and symptoms were relieved in the following 4 months after surgery but recurred 6 months after surgery. The abnormal rhythm of plasma cortisol and ACTH presented periodic secretion and seemingly rose significantly after food intake. EAS was diagnosed according to inferior petrosal sinus sampling (IPSS). Appendiceal mass was identified by ^{68}Ga-DOTA-Tyr3-octreotate (DOTATATE)-PET-CT and removed. The pathological result was consistent with appendiceal neuroendocrine tumor with ACTH (+). The literature review demonstrated 7 cases diagnosed with EAS caused by appendiceal neuroendocrine tumor with similarities and differences.

Conclusion: The diagnosis of an ectopic ACTH-producing tumor caused by the appendiceal neuroendocrine tumor can be a challenging procedure. Periodic ACTH and cortisol secretion may lead to missed diagnosis and misdiagnosis. IPSS is crucial in the diagnosis of EAS and ^{68}Ga-DOTATATE-PET-CT plays an important role in the identification of lesions.

Keywords: Cushing's syndrome, ectopic ACTH syndrome (EAS), cyclic Cushing's syndrome, appendiceal neuroendocrine tumor, ^{68}Ga-DOTATATE-PET-CT, case report

INTRODUCTION

Cushing's syndrome (CS) is a condition of pathological hypercortisolism, which is classified into adrenocorticotropic hormone (ACTH)-dependent and ACTH-independent causes, in which ectopic ACTH syndrome (EAS) belonging to ACTH-dependent CS is a rare cause, which accounts for 5%–10% of CS (1, 2). The typical EAS is characterized by rapidly progressive clinical figures, including fatigue, severe hypokalemia, myasthenia, and serious infection. Therefore, early diagnosis and removal of responsible lesions are crucial, in order to remit hypercortisolism and prevent fatal complications. However, various difficulties may block proper diagnosis and treatment such as cyclic CS or occult EAS.

The common tumors that contributed to EAS had been published including pulmonary or thymic neuroendocrine neoplasms (NENs), pancreatic neuroendocrine tumors (NETs), pheochromocytomas, and medullary thyroid carcinomas (3). Our center had summarized the clinical spectrum of 88 patients diagnosed with EAS, and it was found that thoracic origins (80.7%) were the most common cause of EAS, comprising pulmonary NETs (43.2%, 38/88), thymic/mediastinal NETs (33%, 29/88), and small cell lung cancer (SCLC) (3.4%, 3/88). Pancreatic NETs were found in 6 patients (6.8%) (4). An appendiceal NET is such as rare cause of EAS that there were only seven cases published until now worldwide (5–9).

Here, we report a case of a 34-year-old woman diagnosed with ectopic CS that was caused by excessive ACTH secretion by the appendiceal NET.

CASE REPORT

A 34-year-old female patient was admitted to our hospital due to hypertension and menstrual disorder. Two years before, she gradually developed a moon face, truncal obesity, acne, and hypertension during pregnancy and menopause after delivery. The level of blood potassium was approximately 2.66~3.64 mmol/L (3.5–5.5). She was diagnosed with Cushing's disease in a local hospital depending on elevation levels of 24-h urinary free cortisol (UFC) of 1,155.4 μg (12.3–103.5) and ACTH of 18.2 pg/ml (0–46) (**Table 1**) and suspicious pituitary microadenoma in MRI initially. Consequently, a neurosurgical sella exploration was performed. Postoperative pathology did not conform to ACTH pituitary tumors. Nevertheless, the patient presented peeling and improvement of acne and hypokalemia with reduction of ACTH (13.1 pg/ml), plasma cortisol (8.3 μg/dl), and 24-h UFC (104.4 μg) 4 months after surgery (**Table 1**). The remission of hypercortisolemia was considered at that time. However, 6 months later, severe fatigue, hypokalemia, and hyperglycemia reappeared with an elevation of 24-h UFC (358.2 μg) and plasma cortisol of 720 nmol/L (133–537) (**Table 1**). The patient came to our clinic for further treatment. She has no special past or family history. Physical examination showed typical cushingoid features including moon facies, supraclavicular fatty pad, buffalo hump, skin atrophy, wide purple striae, purpura, and hirsutism. Further examination found hypokalemia, hypertension, osteoporosis (multiple rib fractures), and urolithiasis. Endocrine hormone-related examination revealed elevated levels of 24-h UFC (615.7~837.1 μg). The level of 8 a.m. serum cortisol fluctuated between 21.8 and 25.2 μg/dl, and ACTH was between 17.7 and 19.8 pg/ml (**Table 1**). Further examination suggested an abnormal rhythm of plasma cortisol and ACTH, which presented periodic secretion in 4-h cycles and seemingly rose significantly after meals (**Table 2**). A low-dose dexamethasone suppression test (LDDST; 2 mg of dexamethasone test) confirmed the diagnosis of hypercortisolism. A high-dose dexamethasone suppression test (HDDST; 8 mg of dexamethasone test) was not greater than 50% suppression of 24-h UFC from the basal value. Inferior petrosal sinus sampling (IPSS) revealed a central/peripheral ACTH ratio of less than 2 and less than 3 with desmopressin injection. The peripheral desmopressin stimulation test is shown in **Table 2**, which suggested a positive response to desmopressin. Diagnosis of ectopic ACTH syndrome with the cyclic secretion of ACTH (cyclic CS) was possible. In order to localize the source of ACTH secretion, the following were completed with no hint: octreotide scan; contrast-enhanced CT of the chest, abdomen, and pelvis; and ^{18}F-fluorodeoxyglucose (FDG)–positron emission tomography (PET)–CT. Finally, ^{68}Ga-DOTA-Tyr3-octreotate (DOTATATE)-PET-CT was done, and an abnormal increase in radioactivity uptake was observed in the appendix, with a SUVmax of 17.1. Contrast-enhanced CT reconstruction of the small intestine also suggested a mass in the lumen near the blind end of the appendix with obvious enhancement (**Figure 1**).

TABLE 1 | Laboratory results of the patient.

Time	ACTH (pg/ml) (RR: 0–46)			Serum cortisol (μg/dl) (RR: 4–22.3)			24-h UFC (μg)(RR: 12.3–103.5)
	8 a.m.	4 p.m.	Midnight	8 a.m.	4 p.m.	Midnight	
Before surgery	18.2	57.36	56.55	346.4#	764.2#	719.9#	455.7–1115.4
Postoperative day 1	25.61	68.82		684.5#	1158#		
4 months after surgery	13.1			8.3			104.4
10 months after surgery	10.4	13.6	13.1	472#	717.5#	720.1#	
15 months after surgery	25	91.8	/	24.1	52.15	16.34	615.7
15 months after surgery	19.8	65.1	/	25.2	53.88	/	

The rhythm of serum cortisol and ACTH in this patient.
ACTH, adrenocorticotropic hormone; UFC, urinary free cortisol.
#*The unit of serum cortisol is nmol/L; reference range (RR) is 133~537.*

TABLE 2 | Laboratory results of the patient.

Monitoring of serum cortisol for 18 h (μg/dl)									
6 a.m.	**8 a.m.***	**10 a.m.**	**Noon***	**2 p.m.**	**4 p.m.**	**6 p.m.***	**8 p.m.**	**10 p.m.**	**Midnight**
14.6	40.1	45.6	41	31.3	26.4	42.5	34.8	22.1	16.34
Peripheral desmopressin stimulation test									
		0 min	15 min	30 min	45 min	60 min	90 min	120 min	
ACTH (pg/ml)		19.4	23.7	17.9	15.7	13.5	11.9	36.0	
Serum cortisol (μg/dl)		10.3	21.2	21.9	18.2	16.6	14.8	24.4	

Inferior petrosal sinus sampling and desmopressin test (ACTH: pg/ml)						
Time	Periphery	Left inferior petrosal sinus	Right inferior petrosal sinus	Left internal carotid	Right internal carotid	Central/peripheral
0 min	16.5	19.4	20.5	19.8	20.1	1.24
3 min	27.2	41.6	45.5			1.67
5 min	32.0	48.1	48.8			1.53
10 min	42.7	57.2	57.2			1.34

ACTH, adrenocorticotropic hormone.
**The patient just finished food intake.*

The patient had no history of abdominal pain, changes in bowel habits, or rectal bleeding; neither did she have any complaints of flushing or palpitation. Combined with the above examination results, it was considered that the responsible lesion was appendiceal mass, possibly an appendiceal NET. So a laparoscopic appendectomy was performed, and a mass measuring 1.4 cm × 0.6 cm × 0.5 cm was found in the blind side of the appendix (**Figure 1B**). Pathology examination revealed appendiceal NET (G2, mitotic 4/10HPF) invading the muscle layer of the appendix and the surrounding appendiceal tissue, and abnormality was not observed at the incisor margin. *Immunohistochemistry*: CgA (+), Ki-67 (index 3%), SYN (+), TTF-1 (−), CD56 (+), P53 (wild type), ATRX (+), SSTR2 (+), and ACTH (+). Serum cortisol dropped to 2.5 μg/dl, and ACTH decreased below 5 pg/ml the first day after the operation, suggesting remission of ectopic CS. Hydrocortisone replacement was given and gradually tapered down. Hypokalemia was treated and menstruation resumed, with weight loss and peeling 3 months after surgery.

LITERATURE REVIEW OF CUSHING'S SYNDROME CAUSED BY APPENDICEAL NEUROENDOCRINE TUMOR

CS caused by appendiceal NETs is extremely rare. To our knowledge, only seven other cases have been reported until now. Here, we summarized and analyzed the characteristic features of these cases shown in **Table 3**. Interestingly, except for a 15-year-old girl, all of these reported cases were young women in their 20s~30s with typical cushingoid features and weight gain. Five patients showed an appearance of hyperandrogenemia including hirsutism or acne, but only one patient presented abdominal symptoms such as constipation and chronic abdominal pain. Hypertension, diabetes, and severe osteoporosis also have been found. Diagnosis of CS was certified by elevated 24-h UFC and serum cortisol, as well as LDDST. Levels of ACTH measured in five of these patients were all greater than 20 pg/ml, supporting ACTH-dependent CS. Successful IPSS was done in three patients who presented no gradient, supporting ectopic ACTH syndrome. It is difficult to find ectopic lesions, especially in the early disease course when PET-CT was not available. In two cases, the appendiceal lesions were found during exploratory laparotomy for the proposed adrenal resection. In the other four patients, ^{18}F-FDG-PET-CT, DOPA-PET-CT, ^{68}Ga-DOTATE-PET-CT, and ^{99m}Tc HYNIC-TATE-PET-CT were used to identify the responsible lesion in the appendix. All seven patients underwent appendectomies, and four of them underwent hemicolectomy, which resulted in remission of CS. Pathological results were all consistent with appendiceal NETs, and four of them had positive ACTH staining. Immunostaining for ACTH and pro-opiomelanocortin in case 6 was negative, which did not support diagnosis and may suggest that the tumor either was unrelated to the EAS or represents dedifferentiation of the original tumor. Two of seven patients presented periodic cortisol secretion.

DISCUSSION

Appendiceal NETs represent the most common tumor of the appendix, found in 0.2%–0.7% of all appendectomies (12) and accounting for 2%–5% gastrointestinal NETs (13), commonly being identified incidentally during appendectomy performed for appendicitis. Appendiceal NETS are more common in women and are mostly submucosal at the tip of the appendix. They are less likely to cause obstruction and are mostly asymptomatic (13). NETs originate from neuroendocrine cells, secreting different substances such as somatostatin, gastrin, and ACTH. Excess amounts of these substances can lead to various clinical presentations; for instance, excess ACTH secretion induced CS.

Based on this case report and literature review, the diagnosis of CS caused by appendiceal NETs is challenging; even some patients were misdiagnosed to have pituitary ACTH

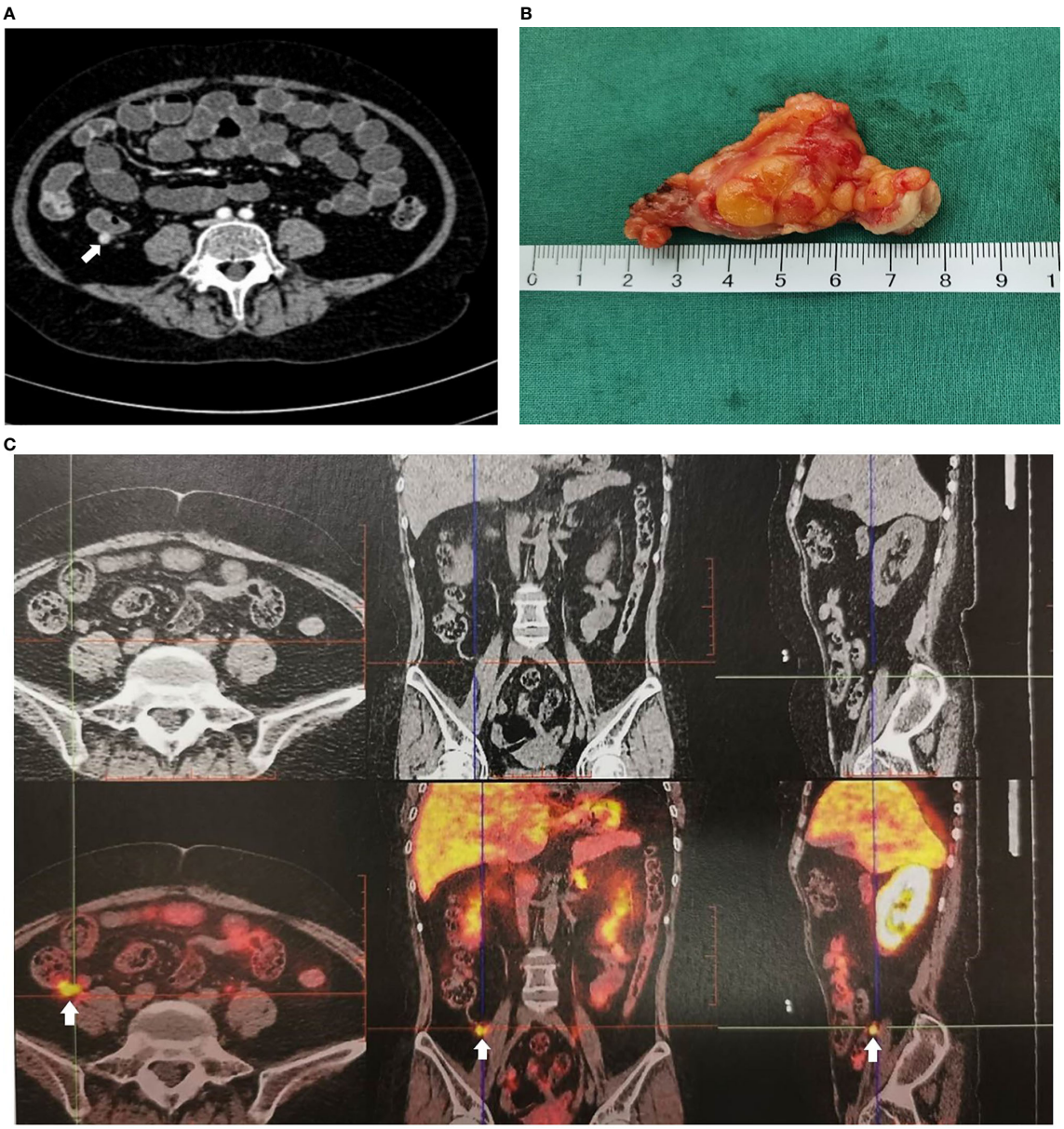

FIGURE 1 | Imaging examination of appendiceal mass. **(A)** Appendiceal mass showed by contrast enhanced CT reconstruction of the small intestine. **(B)** Appendiceal mass. **(C)** Appendiceal mass showed by ^{68}Ga-DOTATATE-PET-CT.

microadenoma, and they underwent pituitary exploration surgery before the discovery of the appendiceal tumor. The main reasons for the difficulty in diagnosis were as follows. First of all, specific clinical manifestations were lacking. Cushing-like appearance, hypertension, diabetes, hypokalemia, and osteoporosis are still the main clinical manifestations. Unexpectedly, abdominal symptoms caused by appendiceal masses were not prominent, and only one patient has relevant symptoms, which was consistent with previous reports on asymptomatic appendiceal NETs (14).

Secondly, it is always challenging to distinguish between Cushing's disease and ectopic ACTH-dependent CS by tests. IPSS has long been the gold standard to reliably exclude ectopic ACTH production but should preferably be performed in a specialized center because of potential patient risk. If IPSS was not allowed, a combination of three or four noninvasive approach tests was recommended, specifically corticotropin-releasing hormone (CRH) and desmopressin stimulation plus MRI, followed by whole-body CT if the diagnosis is equivocal (15). Unfortunately, the hospital where this patient was first admitted did not have the

TABLE 3 | Characteristics of cases with IGSF1 deficiency from published literature.

Author	Case 1 Timothy Miller (5)	Case 2 H. Dobnig (6)	Case 3 David Beddy (7)	Case 4 N. Perakakis (8)	Case 5 Chakra Diwaker (9)	Case 6 Ashley B. Grossman (10)	Case 7 Elżbieta Moszczyńska (11)
Year published	1971	1996	2010	2011	2019	1986	2021
Nation	USA	Germany	USA	Germany	India	UK	Poland
Sex	Female	Female	Female	Female	Female	Female	Female
Age at onset	35	33	23	31	22	24	15
Age at surgery of appendectomy	36	/	23	32	/	44	17
Weight gain	+	+	+	+	/	+	+
Cushingoid features	+	+	+	+	/	+	+
Menstrual disorder	+	+	–	+	/	+	+
Hirsutism or acne	–	+	+	+	/	+	+
Hypertension	+	–	+	–	/	–	–
Diabetes	–	–	–	+	/	–	–
Hypokalemia	+	–	–	–	/	+	+
Osteoporosis	+	+	–	–	/	–	–
Urolithiasis	–	–	–	–	/	–	–
Others	Emotional instability	Swelling	Weakness	Constipation, chronic abdominal pain	/	Edema	Sinus bradycardia
24-h UFC	/	/	1,663 μg (3.5–45)	Elevated	/	/	/
Serum cortisol	10 (5–20)	629 nmol/L (138–689)	31 μg/dl (7–25)	73 μg/dl	/	>2,000 nmol/l	47.1 μg/dl
ACTH	/	6.4 pmol/L (2.2–11)	/	182 pg/ml	8 pmol/L (0–10)	48~204 pg/l	182
Periodic secretion	Not mention	Not mention	Not mention	Not mention	Not mention	+	+ (every 1–2 months)
LDDST	Not suppressed	/	Not suppressed	Not suppressed	Not suppressed	Not suppressed	/
HDDST			+	Suppressed more than 50%	/	Suppressed less than 50%	Suppressed less than 50%
CRH testing	/	No response	/	No response	/	No response	Contradictory
IPSS	/	0.8	No gradient	Not success	1.3:1	Not success	/
Pituitary MRI		+	–	–	–	–	+
Abdominal CT	/	–	2 cm mass medial to the cecum	–	A small lesion from the appendix or cecal mesentery	–	2.4-cm abnormal density in the appendix region
Octreotide scan	/	/	/	–	/	–	/
Fu-PET-CT	/	/	2-cm mass medial to the cecum	–	/	/	/
DOPA-PET-CT/^{99m}Tc HYNIC-TATE	/	/	/	1.8 × 1.1 cm mass in the terminal ileum	1.0 × 1.2 × 1.9 cm (SUVmax, 9.5) in the appendix	/	Increased radiotracer uptake in the appendix
Colonoscopy	/	–	Submucosal mass in the cecum	–	/	–	/
Treatment	Abdominal exploration + appendectomy + adrenalectomy	Appendectomy + right-sided hemicolectomy and lymphadenectomy	Right hemicolectomy	Appendectomy 1 year later operated right hemicolectomy	Laparoscopic appendectomy	Adrenolytic therapy trans-sphenoidal surgery Bilateral adrenalectomy appendectomy with en bloc resection of	Laparoscopic appendectomy Right-sided hemicolectomy

(Continued)

TABLE 3 | Continued

Author	Case 1 Timothy Miller (5)	Case 2 H. Dobnig (6)	Case 3 David Beddy (7)	Case 4 N. Perakakis (8)	Case 5 Chakra Diwaker (9)	Case 6 Ashley B. Grossman (10)	Case 7 Elżbieta Moszczyńska (11)
Pathology examination	Appendiceal carcinoid without invasion	Infiltrating carcinoid tumor	ACTH-producing carcinoid tumor of the appendix	Appendiceal neuroendocrine tumor infiltration of the submucosal and subserosa of the pericolic fat and vascular invasion (G1)	ACTH-secreting appendicular carcinoid	the adherent mesentery Carcinoid tumor with metastases to mesenteric and local nodes	Moderately differentiated NET G2, with metastasis of peritoneum, mesentery, greater omentum, lymph nodes
Histological examination	Positive argentaffinoma reaction	ACTH (+) Neuron-specific enolase (+) Chromogranin (+)	Chromogranin (+) Synaptophysin (+) ACTH (+)	ACTH (+) Chromogranin (+)	/	ACTH (–) Pro-opiomelanocortin (–)	Chromogranin A (+) Synaptophysin (+)
Follow-up	Remission	Remission	Remission	Remission	Remission	/	Remission Recurrence in 7 years

UFC, urinary free cortisol; ACTH, adrenocorticotropic hormone; LDDST, low-dose dexamethasone suppression test; HDDST, high-dose dexamethasone suppression test; CRH, corticotropin-releasing hormone; IPSS, inferior petrosal sinus sampling.

+, the patient had this symptom; –, patient did not have this symptom; /, this result was not mentioned in the report.

capacity to implement IPSS and CRH or desmopressin stimulation tests. Misdiagnosis led to inappropriate treatment options and prolonged illness. In addition, it is difficult to locate space-occupying lesions of the gastrointestinal tract by conventional imaging such as CT. It is gratifying that more and more highly specific imaging techniques are being used in the clinic. Somatostatin receptor scintigraphy (SRS), ^{18}F-FDG, or ^{68}Ga-DOTATATE-PET/CT was considered if CT or MRI did not show typical findings of ectopic tumors (4). Compared to the other two nuclear functional imaging techniques, ^{68}Ga-DOTATATE-PET-CT had a higher affinity for the SSTR2 receptor and higher resolution to illustrate anatomical details, helping in the successful detection and accurate localization of small tumors characterized by SSTR (16–18). Taweesak et al. found that ^{68}Ga-DOTATATE identified the primary ECS in 11/17 (65%) of previously occult tumors (19). The case in our report failed to identify the responsible lesions by CT, octreotide imaging, and ^{18}F-FDG-PET-CT. Finally, ^{68}Ga-DOTATATE-PET/CT successfully located the lesion in the appendix.

Periodic cortisol secretion also contributed to another difficulty in this case. Diagnosis of cyclic CS is based on at least three periods of confirmed hypercortisolemia interspersed by two periods of normocortisolemia (20). Although these criteria apply to most patients, they might be hard to fulfill in others, particularly if due to the long intercyclic phase or intermittent hypercortisolism. The presence of only two peaks and one trough of hypercortisolism, in this case, did not conform to the diagnostic criteria. But given the overall course of the disease, this patient is highly likely to be diagnosed with cyclic Cushing's syndrome. The trough of cortisol production occurred just after sella exploration, giving the false appearance of recovery, which led to an incorrect diagnosis of pituitary ACTH adenoma. Meanwhile, the case showed great increasing levels of serum cortisol and ACTH at 4 p.m. compared to normal levels at 8 a.m., which may result in missed diagnosis of ACTH-dependent hypercortisolism if the blood sample at 4 p.m. was not collected. To further explore the rhythm of cortisol secretion in this patient, levels of serum cortisol were monitored every 2 h (from 8 a.m. to 12 p.m.) and suggested another shorter periodic secretion. Interestingly, most of the peaks seemed to appear after food intake, which had not been reported in previous case reports. A review of 65 reported cases demonstrated that cyclic CS originated in 26% from an ectopic ACTH-producing tumor, which included thymic carcinoid, bronchial carcinoid, pancreatic carcinoid, renal carcinoid, gastric carcinoid, epithelial thymoma, pheochromocytoma, and 2 occult cases. Cycle length ranged from 4 to 180 days (20). In a review of 7 cases of EAS caused by appendiceal carcinoids reported before, two of them showed periodic hormone secretion, but neither of these secretion cycles was as short and related to food consumption as in this case. We speculated that food residues flowing through the intestine and intestinal peristalsis after food intake might stimulate ACTH secretion of appendiceal NET. But it is a pity that we did not extend it for a longer time to confirm the association with food intake to distinguish with a variable secretion of cortisol.

The criteria of the desmopressin test associated with the best compromise between sensitivity and specificity were a relative cortisol increase >18% and ACTH increase >33% for the desmopressin test with 83% sensitivity and 81% specificity for the diagnosis of Cushing's disease (21). This patient presented a positive response to desmopressin, which confirm Cushing's disease, not EAS. However, it has been shown that some cases of EAS expressed the V2 receptor and responded to desmopressin (22). Periodic cortisol secretion may also disturb the result, which can explain the result of this patient.

For most T1 appendiceal tumors confined to the appendix, simple appendectomy is sufficient because of infrequent metastasis. In those with tumors larger than 2 cm, right hemicolectomy is recommended. Controversy exists regarding the management of appendiceal NETs measuring less than 2 cm with more aggressive histologic features. It has been suggested that the presence of lymph node metastases in appendiceal NETs smaller than 2 cm may lead to more aggressive management of appendiceal NETs with adverse prognostic factors (lymphovascular or mesangial invasion or atypical histological features). For these tumors, right hemicolectomy is recommended (23).

In conclusion, eight cases of an EAS due to appendiceal NETs have been reported until now. These cases demonstrate that the diagnosis of an ectopic ACTH-producing tumor can be a challenging procedure, which demands a systemic multidisciplinary approach, regular follow-ups, and the use of various novel imaging techniques. Periodic hormone secretion may be confusing, so there should be careful attention to screening and examination. For occult EAS, gastrointestinal NETs should not be ignored.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YZ contributed to the conception and design of the study, diagnosis and treatment of the patient, literature review, and drafting of the manuscript. WM contributed to the diagnosis and treatment of the patient. YJ contributed to the diagnosis and treatment of the patient. GZ contributed to the treatment of the patient. LW critically revised the manuscript for important intellectual content. FG critically revised the manuscript for important intellectual content. HZ critically revised the manuscript for important intellectual content. LL contributed to the conception and design of the study, and diagnosis and treatment of the patient, and critically revised the manuscript for important intellectual content. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Acromegaly Caused by Ectopic Growth Hormone Releasing Hormone Secretion: A Review

Iga Zendran[1], Gabriela Gut[2], Marcin Kałużny[1], Katarzyna Zawadzka[1] and Marek Bolanowski[1]*

[1] Department of Endocrinology, Diabetes and Isotope Therapy, Wroclaw Medical University, Wroclaw, Poland, [2] Department of Endocrinology, Diabetes and Isotope Therapy, Students research association, Wroclaw Medical University, Wroclaw, Poland

Introduction: Ectopic acromegaly is a rare condition caused most frequently by growth hormone releasing hormone (GHRH) secretion from neuroendocrine tumors. The diagnosis is often difficult to establish as its main symptoms do not differ from those of acromegaly of pituitary origin.

Objectives: To determine most common clinical features and diagnostic challenges in ectopic acromegaly.

Patients and Methods: A search for ectopic acromegaly cases available in literature was performed using PubMed, Cochrane, and MEDline database. In this article, 127 cases of ectopic acromegaly described after GHRH isolation in 1982 are comprehensively reviewed, along with a summary of current state of knowledge on its clinical features, diagnostic methods, and treatment modalities. The most important data were compiled and compared in the tables.

Results: Neuroendocrine tumors were confirmed in 119 out of 121 patients with histopathological evaluation, mostly of lung and pancreatic origin. Clinical manifestation comprise symptoms associated with pituitary hyperplasia, such as headache or visual field disturbances, as well as typical signs of acromegaly. Other endocrinopathies may also be present depending on the tumor type. Definitive diagnosis of ectopic acromegaly requires confirmation of GHRH secretion from a tumor using either histopathological methods or GHRH plasma concentration assessment. Hormonal evaluation was available for 84 patients (66%) and histopathological confirmation for 99 cases (78%). Complete tumor resection was the main treatment method for most patients as it is a treatment of choice due to its highest effectiveness. When not feasible, somatostatin receptor ligands (SRL) therapy is the preferred treatment option. Prognosis is relatively favorable for neuroendocrine GHRH-secreting tumors with high survival rate.

Conclusion: Although ectopic acromegaly remains a rare disease, one should be aware of it as a possible differential diagnosis in patients presenting with additional symptoms or those not responding to classic treatment of acromegaly.

Keywords: acromegaly, ectopic, GHRH, neuroendocrine tumors, pituitary hyperplasia

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****Correspondence:***
Marcin Kałużny
marcin.kaluzny@umw.edu.pl

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INTRODUCTION

Acromegaly is a rare disease with prevalence ranging between 2.8 and 13.7/100,000 persons, mostly caused by a benign pituitary growth hormone (GH) secreting adenoma (1). The term 'ectopic acromegaly' refers to a syndrome caused by secretion of growth hormone releasing hormone (GHRH), or occasionally, by an extra-pituitary source of GH and accounts for less than 1% of all acromegaly cases (2, 3). Ectopic GHRH derives most commonly from functional neuroendocrine tumors (NETs) originating in the lung or the pancreas and results in pituitary hyperplasia and excess GH secretion (4). The term 'ectopic' is used in a broader sense in this review, applying not only to its most common meaning of an abnormal localization, but also basically to the secretion of a hormone by a tissue that does not produce it under physiological circumstances (5). Therefore, GHRH-secreting pituitary gangliocytoma will also be included in the review, as they fall under given definition (6). Ectopic acromegaly clinical features are indistinguishable from those of acromegaly caused by a pituitary somatotropinoma (7). The suspicion of ectopic acromegaly is most commonly drawn when no discrete adenoma is shown in magnetic resonance (MR) imaging of the pituitary gland. Additional manifestations of an extracranial tumor, such as cough or dyspnea for the lung neoplasm or other endocrinopathies for a pancreatic NET, as well as lack of remission of the disease after transsphenoidal adenomectomy may also be suggestive of the disease (3, 8).

PATIENTS AND METHODS

An extensive search was performed for ectopic acromegaly cases described in literature between 1982 and 2021. Research was conducted in PubMed, Cochrane, and MEDline databases using the particular keywords acromegaly, GHRH, ectopic, and neuroendocrine tumors. More than 300 articles were screened. Duplicates, unusable records, and those with partial information were rejected. According to the specific criteria, 127 cases were selected containing confirmation of ectopic GHRH secretion by biochemical and/or histopathological examination, some of them described as a part of case series. GHRH-secreting pituitary gangliocytoma was also included.

Historical Aspects

Since the initial report of the possible association between the neuroendocrine functional tumor and acromegaly, suggested by Altmann et al. in 1959, around 170 cases of suspected ectopic acromegaly have been described in literature to date, mostly as case reports (4, 6, 9–21). To our best knowledge, only 19 cases were described as caused by an ectopic source of GH (22–27). Consequently, the vast majority of the cases were reports of acromegaly due to ectopic GHRH secretion. However, until 1982, when the isolation of GHRH from pancreatic tumors was achieved simultaneously by two research groups, the underlying cause of ectopic acromegaly could only have been suspected (28, 29). To date, 10 cases of possible GHRH secretion by neuroendocrine neoplasms have been reported (7 of the lung, 2 of the pancreas, and one of the foregut origin) and will not be covered in this review (4). Also, 13 cases described between 1984 and 2002 and mentioned in previous reviews will also not be included here due to the lack of records. In this review, 127 cases of acromegaly caused by ectopic GHRH secretion confirmed by biochemical or histopathological examination will be discussed, comprehensively summarizing current knowledge on this rare condition.

Clinical Features

Acromegaly due to ectopic GHRH secretion is more common in women who represent 70% of reported cases (**Table 1**). Patients ranged in age from 14 to 77, with mean age of 43.3 years at the time of diagnosis. The age distribution was comparable for men and women, with mean age of 43.9 and 41.7 years, respectively. The duration of the disease before diagnosis was approximately 7.4 years, which is consistent with the course of acromegaly caused by somatotropinoma (30, 31). Although the age at diagnosis was similar for both men and women, there has been a sex disparity from the time of the onset of symptoms until diagnosis. The diagnostic delay was longer in men by approximately 3.5 years with mean duration of 9.9 years in comparison to 6.2 years in women, which is also in line with pituitary acromegaly characteristics (32).

Tumors Characteristics

Extracranial tumors constituted 78.7% of the cases and the remaining 27 cases presented with GHRH-secreting tumors within the sellar region are shown in **Table 2**. Histopathological evaluation was available for 121 tumors, indicating that 119 of them were neuroendocrine tumors with only 2 exceptions for a pituitary diffuse large B-cell lymphoma (DLBCL) and an adenoid cystic carcinoma of the lung (18, 33). Most common histopathological assessment for tumors of the sellar region was mixed gangliocytoma-pituitary adenoma, representing 77.8% of all intracranial cases. As for the extracranial tumors, the majority of them originated in the lung and pancreas (50% and 35%, respectively), with typical bronchial carcinoid as the most common histopathological diagnosis. Albeit rare, GHRH secretion in other tumors, including pheochromocytoma, lymphoma, paraganglioma, or thymoma has also been reported (4, 11, 15, 34–37). In some cases, tumor resection preceded possible acromegaly development (37–39). According to gross pathology data available for 72 patients, extracranial tumors measured 6.6 cm on average, ranging from 1 to 25 cm. Consequently, most of them were apparent in conventional imaging, with an estimated 86% sensitivity of computed tomography (CT) scan described in the French series of 21 cases (40). The largest pituitary mass found in a case of a mixed gangliocytoma-pituitary adenoma measured 6.5 cm (41). In a few cases, multiple pancreatic tumors were identified (13, 42–44). Gangliocytomas may present a characteristic MRI appearance. Usually these tumors consist of cystic and solid components. Gangliocytomas are hypo- to isointense relative to cortex on T1-weighted images and hyperintense on T2/FLAIR images. The solid portions of the tumors show variable enhancement after gadolinium administration: from none to striking homogeneous

TABLE 1 | Clinical features of 127 patients with GHRH-secreting tumors.

	n	%
Female (F)	89	70.1%
Male (M)	38	29.9%
	Mean ± SD	**Median (range)**
Age (years) (*n=127*)	43.25 ± 14.7	42 (14-77)
Age F (years) (*n=89*)	43.9 ± 14.6	43 (15-77)
Age M (years) (*n=38*)	41.65 ± 15.1	41.5 (14-74)
Duration (years) (*n=68*)	7.4 ± 5.5	6 (1-22)
Duration F (years) (*n=46*)	6.2 ± 4.3	5 (1-22)
Duration M (years) (*n=22*)	9.9 ± 6.8	8.5 (2-20)

enhancement. About one-third of cases contain calcifications, which can be seen on susceptibility weighted imaging (SWI) sequence as low signal structures. There are no signs of hemorrhage or necrosis within the tumor, which may be present in common somatotropinomas. Gangliocytomas can occur anywhere in the central nervous system, however the most common and typical location is the temporal lobe. Other reported sites include the brainstem, sellar region, and spinal cord. At diagnosis, lymph node or distant metastases were present in 42 patients with liver, bones, and lung as the most frequent metastatic site. Presence of metastatic cancerous cells was also reported in the breast, spleen, central nervous system, or heart tissue in isolated cases (33, 40, 45–48). Since lung and pancreas tumors constituted the majority of GHRH-secreting tumors, an additional comparison of their main features and treatment results has been performed in **Table 3**, indicating that pancreatic tumors were associated with multiple endocrine neoplasia 1 (MEN 1) syndrome in over half of the cases and were more inclined to produce other hormones, especially insulin, gastrin, and pancreatic polypeptide (40, 42, 44, 49–52).

Clinical Presentation

Overt acromegaly at different stages was presented by 124 patients, ranging from mild acral enlargement to advanced metabolic complications such as hypertension, diabetes, or hyperparathyroidism, considerably reducing the quality of life (53). Acromegaly symptoms did not differ from those of the classic form of the disease (30, 31, 54). In almost half of the cases, elevated levels of other hormones were observed (**Table 3**). Hyperprolactinemia was the most common symptom associated endocrinopathy which was documented in 44 patients (34.7%) and though usually asymptomatic, it manifested with amenorrhea-galactorrhea syndrome in some patients (36, 55–62). Prolactin hypersecretion derived rather from GHRH-induced pituitary hyperplasia or stalk compression than from the ectopic tumor itself, as its expression was documented immunohistochemically only in intracranial tumor cases. Elevated prolactin levels occur more often in patients with acromegaly caused by an ectopic GHRH source than in those with pituitary acromegaly (30). Less common manifestations included diabetes insipidus (63), Zollinger-Ellison syndrome (49, 51), Cushing syndrome (37, 59, 64), carcinoid syndrome (55), and typical pheochromocytoma symptoms (34), as appropriate for corresponding tumors. MEN 1 syndrome was highly probable in 23 patients based on clinical features. Genetic confirmation of MEN1 mutation was available for 19 tumors, including 18 pancreatic NETs and one thymic carcinoid (13, 36, 40, 42–44, 52, 57, 61, 65, 66). In 4 patients, genetic testing had not been performed. Apart from acromegaly and other features caused by pancreatic tumors, the syndromes included hyperparathyroidism in almost all MEN1 cases (13, 36, 40, 42–44, 52, 57, 61, 65), as well as gonadotroph (42) or mixed PRL-GH (40) secreting pituitary adenomas. A null cell pituitary tumor was also detected in one case (65). Some patients suffered from visual field disturbances

TABLE 2 | Origin of the tumor.

Tumor site (n = 127)		**n**	**%**
Intracranial (n = 27)			
Sellar region		27	100%
Extracranial (n = 100)			
Lung (n = 50)	Typical bronchial carcinoid	43	43%
	Atypical bronchial carcinoid	4	4%
	Other	2	2%
	Lack of histopathological examination	1	1%
Pancreas		35	35%
Gastrointestinal tract		5	5%
Adrenal gland		3	3%
Thymus		2	2%
Liver		1	1%
Mediastinum		1	1%
Retroperitoneum		1	1%
Unidentified		2	2%

TABLE 3 | Lung and pancreatic tumor comparison.

		Lung (*n = 50*)	Pancreas (*n = 35*)
Age at diagnosis (years)	Median (range)	42 (19-77)	37 (14-67)
	Mean ± SD	43 ± 14.3	40 ± 13.9
Diagnostic delay (years)	Median (range)	8 (2-22)	6 (2-19)
	Mean ± SD	8.5 ± 6	7.1 ± 4.8
Tumor size (cm)	Median (range)	5.9 (1.2-10)	6 (1-25)
	Mean ± SD	5.5 ± 2.5	6.7 ± 5
Hyperprolactinemia (%)		30%	57%
Other hormones (%)*	Serum elevated levels	42%	69%
	Immunohistochemistry	8%	40%
Metastases (%)		42%	37%
MEN 1 syndrome		0	18
Remission (full or partial) (%)		78%	69%
Survival rate (%)		94%	86%

**Other hormones include: adrenaline, adrenocorticotropic hormone (ACTH), calcitonin, cortisol, follicle-stimulating hormone (FSH), glucagon, gastrin, insulin, luteinizing hormone (LH), noradrenaline, pancreatic polypeptide (PP), parathormone (PTH), serotonin, somatostatin, thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4), vasoactive intestinal peptide (VIP).*

and severe headaches due to large masses located in the sellar region, mostly macroadenoma or somatotroph hyperplasia (41, 47, 59, 67–72). In 3 cases, acromegaly signs have not been observed despite elevated growth hormone levels (37–39). Taking the typical long course of acromegaly into consideration, it is possible that the disease was diagnosed in its initial stages based on hormone and imaging results, enabling prompt and effective treatment. In some patients, acromegaly symptoms manifested for a long time after surgical treatment of an initially asymptomatic bronchial or pancreatic tumor, due to relapse or remaining metastasis. The longest latency period in these cases lasted as many as 30 years (42, 43, 45, 47, 48, 73–75).

Diagnosis of Ectopic Acromegaly

The most described symptom in cases at preliminary diagnosis was acromegaly, recognized by clinical signs and confirmed with GH level not suppressed after oral glucose tolerance test (OGTT) and/or elevated insulin-like growth factor 1 (IGF-1) concentration. Ectopic source should be suspected when no improvement is observed after pituitary surgery, which was the case in 21 patients (17, 19, 35, 40, 42, 56, 57, 61, 66, 73, 76–82). Other findings leading to ectopic GHRH secretion diagnosis were acromegalic features without any pituitary lesion in magnetic resonance imaging or conversely, acromegaly in the setting of a previously known non-pituitary tumor, sometimes accompanied by other manifestations mentioned above (20.5% and 39.3% of the patients, respectively). In 24 cases of intracranial tumors, ectopic acromegaly was not suspected until the histopathological examination revealed unphysiological expression of GHRH, mostly by gangliocytoma cells (6). Diagnostic criteria of acromegaly, due to GHRH-producing tumor, are met when hypersecretion of GHRH is substantiated in a patient with overt acromegaly and recovery following the resection of corresponding tumor is observed (3, 7). GHRH tumoral secretion can be proved by measuring its plasmatic levels and by positive immunostaining or radioimmunoassay with antibodies anti-GHRH 1-40 and anti-GHRH 1-44 in histopathological examination (3, 83). However, though useful, the immunohistochemical technique is not widely available due to limited access to reagents and tumor tissue which may not always be obtained in the required amount (19). Other less common laboratory methods include hormone extraction (50, 56, 76), measurement of arterio-venous gradient of GHRH across tumoral tissue (57, 84), high performance liquid chromatography (HPLC), or ion exchange chromatography (73, 85) and GHRH mRNA detection with polymerase chain reaction (PCR) (63, 86, 87). Before GHRH isolation in 1982, its possible secretion was tested mostly with bioassay, which is an indirect examination based on GH production by rat pituitary cells triggered by the substance obtained from tumor extracts (88). This method may still serve as an additional test and was recently performed on cultured human pituitary cells (16, 60). As shown in **Table 4**, GHRH expression was documented histopathologically in 99 tumors, mostly by means of immunostaining which was performed in 83 cases. Although GHRH expression by various tumors may be considered more often than it was initially believed, only a small number of patients present overt clinical acromegaly. *In vitro* tests with immunohistochemistry (IHC) and radioimmunoassay (RIA) using anti-GHRH antibodies revealed GHRH expression in up to 14% and 56% (IHC and RIA, respectively) of all type tumors, mostly small cell lung carcinoma, breast carcinoma, and pheochromocytoma, however, acromegalic features were rarely observed (8, 30, 89). This may be due to different reasons, beginning with the possibility that produced GHRH is either not released from the tumor or its amount is not enough to stimulate pituitary cells to produce GH. Moreover, ectopic GHRH activity might be reduced due to abnormal chemical structure or concurrent secretion of somatostatin from tumoral tissue (4, 81). Positive

TABLE 4 | Diagnostic methods used to confirm GHRH-induced acromegaly.

	n	%
GHRH plasma concentration	84	66.1%
Histopathological confirmation of GHRH production	99	78%
Both methods	53	41.7%

immunostaining for hormones other than GHRH was documented in 47 neoplasms; 23 of them were intracranial tumors, mostly mixed gangliocytoma-pituitary adenoma positive for GH and sometimes also PRL. Somatostatin expression has been found in both intra- and extracranial tumor tissues (6 and 9 documented cases, respectively). Other marked substances included ACTH, calcitonin, insulin, gastrin, glucagon, PP, serotonin, and VIP, produced one or more at a time by several tumors (16, 33, 40, 77, 90). As above mentioned, tumoral hormone expression has not always translated to elevated plasmatic levels, let alone the symptoms.

Hormonal Evaluation

GHRH plasma levels were available for 84 patients with a median concentration of 1,273 ng/L, as shown in **Table 5**, along with other growth hormone axis levels. The cutoff suggesting ectopic acromegaly (triggered by GHRH ectopic secretion) is a GHRH level of 300 ng/L according to Scheithauer et al. (91). However, in 2012 Garby et al. proposed the threshold of 250 ng/L as a highly specific marker of an ectopic release of GHRH causing acromegaly based on a series of 21 cases (40). In reviewed cases, 76 out of 81 patients with data available in SI values (93.8%) had GHRH levels above the 250 ng/L cutoff value. Elevated GHRH plasma level (especially >250-300 ng/L), may enable differentiation between ectopic and eutopic acromegaly which usually presents with undetectable GHRH serum concentration (10). Interestingly, in patients with hypothalamic GHRH-secreting tumors, plasma level of this hormone is also low (92, 93). The same laboratory criteria are used to diagnose both pituitary and ectopic acromegaly, namely GH hypersecretion, lack of suppression of GH levels during oral glucose tolerance test (OGTT), and elevated IGF-1 levels (3, 7) and values are shown in **Table 5**. It has been described that ectopic acromegaly is more frequently related with paradoxical serum GH rise (>50% of baseline) after TRH or glucose administration. Another noticed difference was that after GHRH administration, GH rise was attenuated in ectopic acromegaly (7, 30). The most popular commercially available GHRH tests are the enzyme-linked immunosorbent assays (ELISA), which use highly specific antibody-antigen interactions. There are GHRH tests with different sensitivities available for various types of samples. ELISA is the gold standard of immunoassays. Another sensitive but less commercial method of quantifying GHRH in liquid sample is RIA, a method based on radiolabeled antibodies (I^{125}). Some examples for the most commercially available ELISA kits are: Human GHRH ELISA Kit (sandwich ELISA), detecting GHRH in plasma, tissue homogenates, and other biology fluids, with sensitivity < 0.75 pg/mL (Antibodies.com8, United Kingdom; Biorbyt, United Kingdom). Human GHRH ELISA Kit (sandwich ELISA), detecting GHRH in serum, plasma and other biological fluids with sensivity 9.38 pg/mL (Novus Biologicals, LLC, USA; LSBio, USA; Assaygenie, Ireland). Human GHRH ELISA Kit (sandwich ELISA), detecting GHRH in serum, plasma, cell culture supernatants, body fluid, and tissue homogenate with sensitivity 1.0 pg/mL (MyBioSource, Inc.USA). Taking into consideration the poor accessibility of GHRH plasma measurement in some areas of the world, the most common side effect, and relatively large dimensions of the related extracranial tumors, the authors suggest that a chest radiograph and an abdomen ultrasound should be performed in every acromegaly case as a minimum diagnostic procedures in order not to overlook a source of an ectopic production. In some cases abdomen and chest CT should be considered, especially in cases of unequivocal acromegaly diagnosis and the absence of a pituitary tumor in MR imaging.

Pituitary Morphology

Pituitary imaging was available for 114 patients (89.8%), with MR imaging being the most commonly used method. As shown in **Tables 6**, **7**, its interpretation might be difficult and misleading in GHRH-induced acromegaly. Exposure to GHRH hypersecretion often leads to pituitary somatotroph hyperplasia, which is considered to be a characteristic feature of ectopic acromegaly. Apart from this, somatotroph hyperplasia has only been found in patients with McCune-Albright syndrome (4). However, studies show that pituitary adenoma may also occur in the course of ectopic GHRH secretion when the exposure is prolonged which was proved in laboratory transgenic mice (94). Although rare, such cases have also been described in patients, however it has not been proved that the adenoma was due to GHRH secretion and not incidental (40, 65, 77). The association has also been shown for sellar region gangliocytoma where the proximity of GHRH source may result in paracrine stimulation of pituitary cells eventually leading to adenomatous transformation (41, 62, 95). It has been suggested that somatotroph transformation in response to ectopic GHRH may exhibit a continuum model of transformation rather than a surge character of changes and therefore both hyperplasia and adenomatous cells may be present at the same time (57, 96, 97). Hence, proper distinction between pituitary adenoma and hyperplasia is not always achievable by means of imaging methods. This may, in consequence, lead to unnecessary surgical resection, especially when there is no other indication of an extracranial neoplasm as an underlying cause of acromegaly. Pituitary surgery *via* transsphenoidal approach or craniotomy was

TABLE 5 | Serum/plasma concentrations of hormones in the hypothalamic–pituitary–somatotropic axis (HPS axis) in patients with ectopic acromegaly.

		n	Mean ± SD	Median (range)
GHRH (ng/L)		81	8,965 ± 19,547	1,273 (60.1-145,000)
GH (ng/mL)	Basal	94	54.2 ± 83.7	29.6 (1.7-488)
	OGTT Nadir	66	43.1 ± 60.6	25.4 (0.1-323)
	Maximum value in TRH test	31	178 ± 272	66.5 (6.9-1,264)
	Post GHRH test	7	33.2 ± 21.9	33.2 (17.7-487)
IGF-1*		84	2.7 ± 1.5	2.4 (1-11.73)

**expressed as patient's IGF-1 value/ULN (upper limit of normal).*

TABLE 6 | Pituitary imaging and histopathology of intracranial GHRH-secreting tumors.

	n	%
Imaging *(n = 18)*		
Adenoma	12	66.7%
Unclear lesion	5	27.8%
Enlargement	1	5.5%
Histopathology *(n = 27)*		
Mixed gangliocytoma-pituitary adenoma	21	77.8%
Gangliocytoma	3	11.1%
Adenoma	1	3.7%
Lymphoma with somatotroph hyperplasia	1	3.7%
Normal	1	3.7%

performed in 54 patients. In 28 cases it appeared to be curative, as the pituitary tumor has been found to cause acromegaly. In an additional 5 patients it served as symptomatic treatment of bitemporal hemianopsia or headache induced by the pituitary mass. However, in 21 cases (38.9% of performed procedures), resection was proved not to be necessary, revealing hyperplasia or normal pituitary tissue in histopathological examination and no improvement afterwards. This stresses the need for a cautious approach while interpreting pituitary images and proves there is necessity for accurate hormonal evaluation, especially in ambiguous cases, in order to avoid unnecessary surgery. In patients with extracranial ectopic source of GHRH, sellar enlargement proved to be reversible in many cases and pituitary size diminished significantly after resection of the primary tumor or somatostatin receptor ligands (SRL) therapy (21, 49, 52, 58, 68, 98).

Treatment Strategies and Prognosis

Despite various treatment modalities being used in ectopic acromegaly management, tumor surgical resection remains a method of choice due to its highest effectiveness and should be performed when feasible (3). Complete tumor resection was the main treatment method for 85 patients, including adenomectomy in 26 of 27 intracranial tumors cases. In a few cases, surgical resection of liver metastases was performed with satisfactory results (40). SRL therapy is the preferred treatment option for patients with inoperable tumors or disseminated metastatic disease, yet, it may also serve successfully as an adjuvant therapy for patients who undergo surgical procedures (3). Therapy with SRL was administered to 37.8% of patients at different stages of treatment, being the main method in 22 cases (9, 40, 45, 47, 50, 68, 75, 99, 100). However, in some cases it was proved to not be sufficiently effective, presumably due to the lack of somatostatin receptor 2 (SSTR-2) in tumor tissue, which is also uncommon, in hyperplastic somatotroph cells (61). In other cases, even if somatostatin receptors were not found in tumor tissue, SRL therapy showed at least partial efficacy due to typical SSTR expression in somatotroph cells (61). In the other cases it appeared to be the only successful method even after surgical treatment (78). The efficacy of surgical resection and SRL therapy cannot be righteously compared in this condition, as the second method has usually served as basic treatment in more advanced cases, often with metastatic disease which itself is a factor of worse prognosis (36). Along with SRL, other supportive methods that may be mentioned are chemotherapy, radiotherapy, immunotherapy, metastases embolization, and other hormonal treatments such as bromocriptine or pegvisomant (9, 18, 33, 36, 40, 42, 43, 46, 49, 51, 73, 78, 90, 96). Altogether, 41 patients received one of these adjuvant treatment options, with pituitary radiation being the most frequent modality (18, 19, 40, 59, 64, 67, 70, 77, 78, 96). Therapeutic results were available for 115 patients with an overall survival rate of 88.7%. Complete and partial remission following treatment was documented in 47.8% and 28.7%, respectively. As for patients with metastatic tumors, their overall survival rate did not differ much from patients with non-metastatic tumors, with outcomes of 83% and 92%, respectively. Nevertheless, metastatic disease appears to be a factor of treatment with worse results, as full or partial recovery was documented for 87.8% of patients without metastases and only 56.1% with disseminated tumors. Relapse and progression were also observed much more frequently within the latter (40, 57, 91). Mean follow-up was 4.2 ± 5.2 years for all 81 patients with available data. This outcome is in accordance with the literature data indicating that prognosis in neuroendocrine tumors is rather favorable even if diagnosed at a metastatic stage and the course of disease remains indolent in most cases (101, 102). Recovery was defined by improvement of symptoms as well as normalization in GH and IGF-1 serum levels. Full GHRH normalization is achievable after surgical treatment but not with SRL therapy. Hormonal treatment appears to reduce GHRH level but not below the detection limit (10). Evaluation of plasma GHRH may be considered useful when anticipating relapse as the rise of GHRH concentration can occur before recurrence of clinical manifestations (11).

CONCLUSION

Acromegaly caused by GHRH release is very rare disease, although one should be aware it exists. Signs, symptoms, and common hormonal evaluation do not differentiate clearly between tumors secreting GH and GHRH. Pituitary imaging does not provide proper diagnosis between pituitary adenoma and hypertrophy and could result in unnecessary surgery. GHRH tumor secretion can

TABLE 7 | Pituitary imaging and histopathology of extracranial GHRH-secreting tumors.

	n	%
Imaging *(n = 96)*		
Enlargement	43	44.8%
Adenoma	20	20.8%
Normal	18	18.8%
Unclear lesion	10	10.4%
Empty sella	3	3.1%
Microcystic lesion	2	2.1%
Histopathology *(n = 29)*		
Hyperplasia	20	69%
Adenoma	3	10.3%
Mixed adenoma and hyperplasia	3	10.3%
Other	2	6.9%
Normal	1	3.5%

be proved by measuring its plasmatic levels and using histopathological methods with immunohistochemical techniques, however, these are still hardly accessible procedures. GHRH level above 250 ng/L indicates a high probability of an ectopic cause of acromegaly. Vast majority of GHRH-producing tumors are neuroendocrine tumors, quite often associated with MEN-1 syndrome.

AUTHOR CONTRIBUTIONS

Idea for the article: MB, MK, and KZ; literature search and data analysis: IZ and GG, drafting the manuscript: MK, IZ, and GG; supervision and critical revision of the work: MB, MK, and KZ. All authors contributed to manuscript revision, read, and approved the submitted version.

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Aviral Singh,
GenesisCare Australia Pty Ltd, Australia

REVIEWED BY
Vineet Pant,
Royal Liverpool University Hospital, United Kingdom
Manoj Gupta,
Rajiv Gandhi Cancer Institute and Research Centre, India
Osborn Jiang,
University of New South Wales, Australia

*CORRESPONDENCE
Ivana Simic Vukomanovic
drivanasimic@gmail.com

†These authors have contributed equally to this work and share first authorship

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Predicting the survival probability of functional neuroendocrine tumors treated with peptide receptor radionuclide therapy: Serbian experience

Vladimir Vukomanovic[1,2†], Katarina Vuleta Nedic[1,2], Marija Zivkovic Radojevic[1,3], Aleksandar Dagovic[1,4], Neda Milosavljevic[1,3], Marina Markovic[4,5], Vladimir Ignjatovic[5,6], Ivana Simic Vukomanovic[7,8*], Svetlana Djukic[5,9], Marijana Sreckovic[10], Milena Backovic[11], Marko Vuleta[12], Aleksandar Djukic[13,14], Verica Vukicevic[15] and Vesna Ignjatovic[1,2†]

[1]Department of Nuclear Medicine and Clinical Oncology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia, [2]Department for Nuclear Medicine, University Clinical Center Kragujevac, Kragujevac, Serbia, [3]Department for Radiotherapy, University Clinical Center Kragujevac, Kragujevac, Serbia, [4]Department for Medical Oncology, University Clinical Center Kragujevac, Kragujevac, Serbia, [5]Department of Internal Medicine, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia, [6]Clinic for Cardiology, University Clinical Center Kragujevac, Kragujevac, Serbia, [7]Department of Social Medicine, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia, [8]Department of Health Promotion, Institute of Public Health, Kragujevac, Serbia, [9]Clinic for Hematology, University Clinical Center Kragujevac, Kragujevac, Serbia, [10]Department of Medical and Business-Technological, Academy of Professional Studies Sabac, Sabac, Serbia, [11]Department for Pathology, Faculty of Medicine, University of Belgrade, Belgrade, Serbia, [12]Department for Cardiology, Clinical Hospital Center "Dr Dragisa Misovic Dedinje", Belgrade, Serbia, [13]Department of Pathophysiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia, [14]Clinic for Endocrinology, Diabetes and Metabolic Diseases, University Clinical Center Kragujevac, Kragujevac, Serbia, [15]Emergency Medical Institute, Belgrade, Serbia

Introduction: Peptide receptor radionuclide therapy (PRRT) is a treatment option for well-differentiated, somatostatin receptor positive, unresectable or/and metastatic neuroendocrine tumors (NETs). Although high disease control rates seen with PRRT a significant number NET patients have a short progression-free interval, and currently, there is a deficiency of effective biomarkers to pre-identify these patients. This study is aimed at determining the prognostic significance of biomarkers on survival of patients with NETs in initial PRRT treatment.

Methodology: We retrospectively analyzed 51 patients with NETs treated with PRRT at the Department for nuclear medicine, University Clinical Center Kragujevac, Serbia, with a five-year follow-up. Eligible patients with confirmed inoperable NETs, were retrospectively evaluated hematological, blood-based inflammatory markers, biochemical markers and clinical characteristics on disease progression. In accordance with the progression og the disease, the patients were divided into two groups: progression group (n=18) and a non-progression group (n=33). Clinical data were compared between the two groups.

Results: A total of 51 patients (Md=60, age 25-75 years) were treated with PRRT, of whom 29 (56.86%) demonstrated stable disease, 4 (7.84%) demonstrated a partial response, and 14 (27.46%) demonstrated progressive disease and death was recorded in 4 (7.84%) patients. The mean PFS was a 36.22 months (95% CI 30.14-42.29) and the mean OS was 44.68 months (95% CI 37.40-51.97). Univariate logistic regression analysis displayed that age ($p<0.05$), functional tumors ($p<0.05$), absolute neutrophil count ($p<0.05$), neutrophil-lymphocyte ratio-NLR ($p<0.05$), C-reactive protein-CRP ($p<0.05$), CRP/Albumin ($p<0.05$), alanine aminotransferase-ALT ($p<0.05$), were risk factors for disease progression. Multivariate logistic regression analysis exhibited that functional tumors ($p<0.001$), age ($p<0.05$), CRP ($p<0.05$), and ALT ($p<0.05$), were independent risk factors for the disease progression in patients with NETs. Tumor functionality was the most powerful prognostic factor. The median PFS (11.86 ± 1.41 vs. 43.38 ± 3.16 months; $p=0.001$) and OS (21.81 ± 2.70 vs 53.86 ± 3.70, $p=0.001$) were significantly shorter in patients with functional than non-functional NETs respectively.

Conclusion: The study's results suggest that tumor functionality, and certain biomarkers may serve as prognostic survival indicators for patients with NETs undergoing PRRT. The findings can potentially help to identify patients who are at higher risk of disease progression and tailor treatment strategies accordingly.

KEYWORDS

NET, PRRT, functional tumors, overall survival, progression free survival

1 Introduction

Neuroendocrine tumors (NETs) are a heterogenous group of tumors originating from widely distributed neuroendocrine cells that have both "neuro" and "endocrine" features (1). This entity with a broad spectrum of clinical manifestations and complex histopathological characteristics differs in grade, differentiation, functional status, and primary site (2). Although the biological behavior of the majority of well-differentiated NETs is relatively indolent, others may be more aggressive and associated with poor prognosis (3). Over the past few decades, peptide receptor radionuclide therapy (PRRT) with radiolabeled somatostatin analogs (SSAs) has gain momentum in the management of inoperable or metastatic, well-differentiated NETs that express somatostatin receptors (SSTR). The range of indication for PRRT was expanded overtime, from gastroenteropancreatic (GEP) NETs to the treatment of SSR positive bronchopulmonary NETs (BP-NETs), paraganglioma and medullary thyroid cancers (4–6). It was shown in NETTER-1 trial that PRRT plus long-acting octreotide improve progression-free survival (PFS) and overall survival (OS) in advanced midgut NETs in comparison to high dose of long-acting octreotide treatment alone (7). Despite high disease control rates seen with PRRT, a subset of the NET population will not respond to radionuclide therapy or even disease progression will be registered (8). Therefore, in order to predict the anti-tumor effect of PRRT, it is necessary to determine reliable response predictors including clinical parameters, biomarkers or imaging (9). Recently, it has been more obvious that inflammatory response also affects tumor growth and patient outcomes (10). Several studies have pointed out the prognostic role of hematological and other blood-based markers of inflammation, including neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR), CRP/albumin ratio (CRP/Alb) in treatment outcomes of patients receiving 177-Lu based PRRT (9–11). The objective of this study is to evaluate the prognostic abilities of inflammatory and other clinical markers in patients with neuroendocrine tumors who are initiating PRRT.

2 Materials and methods

This retrospective study included 51 NET patients who received PRRT in the University Clinical Center Kragujevac, Serbia, covering a 5-year follow-up period (2018-2023). All patients were evaluated and determined to be eligible for PRRT by a dedicated NETs Tumor Board at the University Clinical Center Serbia, Belgrade. The main inclusion criteria were pathologically and clinically confirmed NET with positive SSTR-based imaging (^{99m}Tc-HYNIC-TOC). Patients with autoimmune diseases and other primary tumors were excluded. The Ethics Committee of the University Clinical Center Kragujevac approved the study (01/23-132).

Patients data as age, gender, tumor localization, pathological findings (WHO classification, tumor size, lymph node metastasis, histological grade, and mitosis), and distant metastasis were collected from the electronic medical records system. Peripheral blood tests (blood count, liver and kidney function, albumin level, CRP, hormonal secretion) were performed before first PRRT. Based on their origin, NETs were categorized into three groups: GEP, lungs, and other organs. Tumor grade was classified as grade 1, grade 2, or grade 3 (12). Tumor functionality was assessed based on the presence of typical clinical symptoms associated with carcinoid syndrome (facial flushing, abdominal pain, diarrhea, bronchospasm) and elevated 24-hour urine levels of 5-hydroxyindoleacetic acid (5-HIAA), chromogranin (CgA) and NSE (13).

There was an interval of 4-6 weeks between the use of long-acting SSA and PRRT. PRRT was administered following a standardized Lu-177 based protocol with a dosage of 5.55 GBq per cycle. The cycles were repeated at intervals of 8-12 weeks, mostly DOTA-octreotate based SSA with median cumulative activity of 22 GBq, median four cycles. Renal protection with an amino acid-based solution was administered during the PRRT treatment.

2.1 Determination of biochemical and hematological parameters

The concentrations of biochemical markers were measured using standard methods in Laboratory diagnostic service of the University Clinical Center Kragujevac. Serum concentrations of ferritin, C-reactive protein-CRP, aspartate aminotransferase-AST, alanine aminotransferase-ALT, creatine kinase-CK, lactate dehydrogenase-LDH, renal function test (urea and creatinine) were determined by the reagents (Beckman Coulter Inc. Brea, USA) certified and validated for the use on Olympus AU680 Analyzer. Using the blood count results: platelets and absolute counts of white blood cells subtypes (neutrophils, monocytes, lymphocytes), the indices were computed: platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR), and systemic inflammation response index (SIRI). SIRI was defined as multiplication of neutrophils and monocytes divided by lymphocytes count. We assessed CRP to albumin ratio (CRP/Alb) by dividing CRP in mg/L through albumin in g/L. Plasma chromogranin A (CgA) was assessed by ELISA kit, serum neuron-specific enolase-NSE was measured by an immunoradiometric assay (IRMA), 5-hydroxy-3-indoleacetic acid-5-HIAA was measured in 24-hour excreted urine by ELISA.

2.2 Follow-up

Evaluation of response to therapy was done using contrast-enhanced MDCT or MRI (4-8 weeks after 2 applied cycles). The results of PRRT were interpreted according to RECIST 1.1. According to the response to the therapy, the patients were divided into two groups, the group with progression (PD) and the group without progression (SD or PR). The primary endpoints were overall survival (OS) and Progression free survival (PFS). OS was defined as the interval between the date of first PRRT and death from any cause. PFS was defined as the time from the date of the first-PRRT to the time of the disease progression.

2.3 Statistics

The collected data underwent descriptive statistical analysis methods. Significance of difference for continuous variables was assessed using the parametric Student's t-test and, in the case of non-normal data distribution, nonparametric tests such as the Mann–Whitney U test were employed. Categorical variables were analyzed using the $\chi 2$ test. Statistical significance was determined when the probability of the null hypothesis was less than 5% ($p<0.05$). Variables that marked as significant predictors for disease progression in univariate logistic analysis were subsequently subjected to multivariate binary logistic regression. To control for false discovery rate in multiple comparisons, the Benjamini–Hochberg method was applied for p-value correction. The length of survival was evaluated using the Kaplan-Meier method, while differences between groups were assessed using the log-rank test. SPSS-20 statistical software for Windows was used to calculate and process the data (Chicago, IL, USA).

3 Results

Study included 51 patients with a mean age of 59.83 ± 10.83 years, median 60 years (range 25–75) at enrollment. Among those enrolled, 26 (50.98%) were female and 25 (49.02%) were male, and 84.70% of the patients were in good health (ECOG performance status) (14). GEP-NETs were the most common primary tumors (52.94%), BP-NETs were being present in 21.56%, and others were unknown primary origin. There were 31 (60.78%) non-functioning NETs and 20 (39.22%) functioning NETs. Based on the Ki-67 proliferation index of the tumor, predominantly disease grade was G2 (45.10%), compared to G1 (27.45%) and G3 (27.45%). Other baseline characteristics are shown in Table 1. Long acting somatostatin analogues were administered to 48 (94.11%) patients.

The results of our study show that the five year overall survival is 84.31%. At the time of the analysis, the median OS for both groups had not been reached, while the mean OS was 44.68 months (95% CI 37.40-51.97). Mean value of PFS was a 36.22 months (95% CI 30.14-42.29). After the introduction phase, the vast of the patients (72.47%) achieved control of the disease with SD verified in 29 (56.86%) patients and PR found in 4 (7.84%) patients, by RECIST 1.1 criteria. However, 14 (27.46%) patients had PD and death was recorded in 4 (7.84%) patients. Complete response was not observed during the five-year follow-up. The PRRT was commonly tolerated well and no grade 3 and 4 toxicity was reported, based on the National Cancer Institute Common Terminology Criteria for Adverse Events-CTCAE, version 5.0.

Univariate analysis showed that, among all variables, only 7 parameters had a statistically significant impact on the progression onset (age, functional tumors, absolute neutrophil count, NLR, CRP, CRP/Albumin, ALT). Variables that had been demonstrated the statistically significance ($p<0.05$) according to univariate

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TABLE 1 Analysis of risk factors for disease progression in patients with NETs treated with PRRT.

Variables	Progression group (n=18)	Control group (n=33)	Test and p value
Age (year)	64.10 ± 9.46	55.56 ± 12.21	Z=-1.898*** p=0.030; p=0.007#
Non-functional tumors	6	25	χ^2= 7.892** p=0.009; p=0.014#
Functional tumors	12	8	
Female	11	15	χ^2= 0.759** p>0.05
Male	7	18	
GEP-NET	7	20	χ^2= 1.357** p>0.05
Lung-NET	6	5	
Unknown primary origin	5	8	
NET G1	9	5	χ^2= 1.420** *p*>0.05
NET G2	7	16	
NET G3	2	12	
Hynic-TOC Krenning score <3	14	5	χ^2= 1.820** *p*=0.403
Hynic-TOC Krenning score ≥3	4	28	
Capecitabine–Temozolomide Chemotherapy before PRRT	16	8	χ^2= 0.759** p>0.05
Ki-67 (%)	19.32 ± 14.55	17.16 ± 8.90	t=0.394* p>0.05
RDW (%) (red cell distribution width)	15.16 ± 1.99	14.98 ± 2.23	t=-0.196* p>0.05
Erythrocytes (10^{12}/L)	4.36 ± 0.55	4.35 ± 0.66	t=0.020* p>0.05
WBC (10^9/L) (white blood cell count)	6.81 ± 1,89	7.1 ± 2.75	t=0.347* p>0.05
Absolute neutrophil count (10^9/L)	5.09 ± 2.34	3.25 ± 1.20	Z=2.110*** p=0.044; p=0.021#
Absolute lymphocyte count (10^9/L)	1.60 ± 0.81	1.98 ± 1.18	t=-0.781* p>0.05
NLR (neutrophil-lymphocyte ratio)	4.66 ± 4.16	1.78 ± 1.08	t=2.397* p=0.024; p=0.028#
Absolute monocyte count (10^9/L)	0.73 ± 0.47	0.64 ± 0.46	t=-0.495* p>0.05
Platelets (10^9/L)	231.22 ± 66.91	228.17 ± 76.51	t=-0.111* p>0.05
SIRI (systemic inflammation response index)	4.85 ± 8.38	1.52 ± 2.22	t=0.589* p =0,058
PLR (platelet-lymphocyte ratio)	226.62 ± 259.96	108.40 ± 73.93	t=0.681* p>0.05
MPV (IU/L)	9.81 ± 0.74	9.59 ± 1.61	t=-0.370* p>0.05
CRP (mg/L)	11.84 ± 9.27	3.36 ± 3.14	t=2.117* p=0.044; p=0.040#
CRP/Alb	0.34 ± 0.23	0.12 ± 0.09	t=2.607* p=0.017; p=0.049#
Albumin (g/L)	41.36 ± 444	42.77 ± 4.30	t=0.769* p>0.05

Data represent the mean value ± 1 standard deviation.
*Student's t-test for independent samples.
**χ^2 test.
***Mann–Whitney U test.
#Benjamini-Hochberg method of correction of unadjusted p values.

analysis (Table 1), were further analyzed using multivariate logistic regression. Multivariate regression analysis emphasized that age, functionality, ALT and CRP are an independent risk factor for shorter PFS (Table 2).

Furthermore, multivariate regression analysis indicated that tumor functionality was the most powerful prognostic factor on the appearance of progression (p<0.001). The Figure 1 presents that functional NETs have a lower PFS and OS. Kaplan–Meier analysis showed that for the study group, the median PFS was significantly shorter in patients with functional (11.86 ± 1.41, median 10, 95% CI: 9.06-14.64 months) than non-functional tumors (43.38 ± 3.16, 95% CI: 37.18-49.58 months), with statistical significance of p=0.001 (Mantel-Cox), HR 0.188 (0.069-0.516). In addition, OS was inversely related to tumor functionality too. In the subgroup analysis, median OS was shorter (p=0,001, Mantel-Cox) in patients with functional tumors (21.81 ± 2.70, median 25, 95% CI: 16.51-27.11 months) contrast to non-functional tumors (53.86 ± 3.70, 95% CI: 46.61-61.12 months), HR 0.155 (0.053-0.458).

4 Discussion

In this study, we analyzed the prediction of survival in patients treated with PRRT therapy in a cohort of 51patients with well-differentiated NETs from different sites of primary origin.

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TABLE 2 Multivariate binary logistic regression for disease progression factors in patients with NETs treated with PRRT.

Variables	Odds ratio(95% CI)	P value
Age (year)	1.191 (0.930-1.525)	p=0.027
Functional tumors	181.56 (0,081-404833,98)	p<0.001
Absolute neutrophil count	7.993 (0.011-6076.18)	p=0.566
NLR (10^9/L)	0.327 (0.006-16.844)	p=0.811
ALT (IU/L)	1.770 (0.445-1.334)	p=0.046
CRP (mg/L)	1.760 (0.374-1.544)	p=0.005
CRP/Albumin	46.518 (0.000-5.634)	p=0.834

According to the literature, the median age at diagnosis of NETs typically falls within the range of 61-69 years (3, 15–19). Consistent with these findings, our study also revealed a similar pattern, with mean age of 59.83 ± 10.83. We further demonstrated that age had a statistically significant hazard ratio (1.191, 95% CI 0.930-1.525, p=0.027) affecting survival, suggesting that NETs may be more progressive in older individuals compared to younger ones. Specifically, it has been reported that patients over 40 years of age have an increased risk of death (15–17), although the clinical benefit of PRRT is satisfactory in both older and younger patients (16). In the literature, it has been shown that females tend to have better survival compared to males in the context of NETs (18, 19). However, our study did not find a significant association between gender and survival outcomes. In this study, about a half (45.10%) of NETs were G2, followed by G1 and G3 (27.45% respectively), based on the 2017 WHO criteria (12). We found that the higher the grade of the NETs were more associated with the poorer prognosis, without statistical significance between groups.

Predictive factors of PRRT response are lacking. Here, we aimed to identify predictors of treatment response by evaluating chronic inflammation markers. Chronic inflammation play an important role in the proliferation of malignant cells, angiogenesis, and metastasis of NETs and other neoplasms (8, 10, 20–26). There is increasing evidence that markers of inflammation can be used for the prognostic evaluation of various malignant tumors, including NETs. Inflammatory indexes in the blood, like NLR and PLR, are low cost, easily feasible, and can be measured repeatedly (10, 20–25). The study revealed that patients with disease progression had significantly higher levels of neutrophils, CRP, CRP/Alb and NLR. The secretion of growth factors from malignant cells causes the increased number of neutrophils in cancer patients. Additionally, neutrophils secrete cytokines that can impact the proliferation, spread, and metastasis of tumor cells (21).

Previous studies evaluated inflammatory markers like PLR, SIRI, CRP/Alb ratio, and showed that the high NLR and PLR significantly correlated with worse PFS and OS (20–25). Univariate analysis revealed that patients with an increased neutrophils count, high NLR, CRP, CRP/Alb, ALT, older patients and patients with functional NET had shorter OS and DFS. A high NLR and CRP most likely reflects an inadequate immune response that does not eliminate the tumor, but creates an environment suitable for its growth. Although the levels of these markers were higher in the study group, multivariate analysis demonstrated that only age, functionality, ALT and CRP remained significant as independent prognostic factor for disease progression and survival. ALT and AST transaminasis, reflecting the grade of liver impairment. The detected significance of ALT serum levels can be explained by reflecting liver involvement. In the literature, patients with normal ALT level had a longer PFS, suggesting that the levels of liver transaminases have a guiding effect on prognosis (26, 27).

Chromogranin A had been used as a valuable tumor marker in NETs and elevated levels of CgA and 5-HIAA as well, has previously been associated with poor prognosis was associated with poor outcome (28, 29). In the current patient population, CgA, 5-HIAA and NSE levels was not found to significantly affect survival, although higher levels of these biomarker were noticed in progression group.

The reported 5-year overall survival of 84.31% in the current study cohort was within the reported range in the literature (30–32). Mean PFS and OS in our study was 36.22 and 44.68 months respectively, which is slightly lower compared to values demonstrated in other

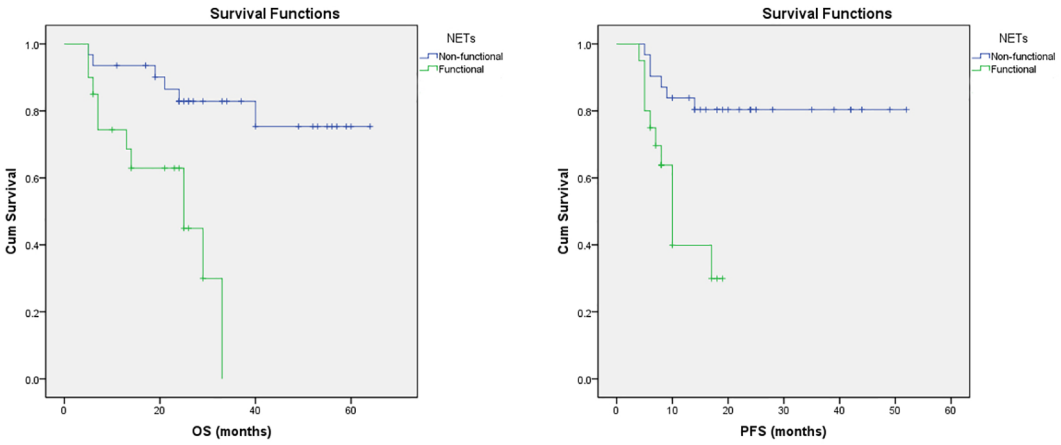

FIGURE 1
Survival trends in patients with NETs. Kaplan–Meier curves of OS and PFS in patients with NETs treated with PRRT based on their functional status.

studies (29, 32). Differences between the current study and the literature exist due to enrollment of lung and G3 NETs, treated with PRRT. The patients with GEP-NET are known to have a much better survival than patients with primary lung NETs. Other studies included only patients with G1 and/or G2 tumors, who probably have a longer OS than patients with G3 grade tumors (33).

The majority NETs are non-functional, as reported in this study (60.78%) and in the literature (60-90%) (8). Functional NETs are known to have a wide spectrum of biological and/or growth behavior. Therefore, management of functional types of NETs is very complex and remains an unmet clinical challenge. Treatment strategy often depends on the presence of various symptoms, grade of the tumor, and clinical stage (34, 35).

As shown in our series, the presence of functional NETs are associated with poor OS and PFS, respectively (Figure 1). Also, non-functioning tumors may alter behavior and/or become functioning and perception of this is essential concerning the strategies for the treatment options. This adds to our knowledge about PRRT in various NET groups and may help when assessing who can benefit from PRRT therapy.

In conclusion, NETs are heterogeneous group of neoplasms that could be treated with various therapeutic approach. We demonstrate that patients with well-differentiated NETs treated with PRRT, the existence of functional tumors is the major independent predictor for survival outcomes. Additionally, age, ALT, CRP, are useful independent risk factor for predicting survival in patients with NETs.

4.1 Limitations of study

This study had its limitations. The current series was based on a relatively small sample size, which was performed retrospectively and the heterogeneity of the patients population. However, the low incidence of NETs is well-known and the number of patients treated PRRT, so this limitation applies to many studies in the field.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The studies involving humans were approved by Ethics Committee of the University Clinical Center Kragujevac (01/23-132). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

VlV: Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis, Project administration, Investigation, Methodology, Supervision, Validation. KV: Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Validation. MZ: Data curation, Formal analysis, Software, Validation, Writing – review & editing, Methodology, Writing – original draft. ADa: Conceptualization, Data curation, Resources, Supervision, Validation, Writing – review & editing. NM: Conceptualization, Data curation, Formal analysis, Visualization, Writing – review & editing. MM: Conceptualization, Formal analysis, Supervision, Validation, Writing – review & editing. VlI: Data curation, Formal analysis, Software, Supervision, Validation, Visualization, Writing – review & editing. IS: Conceptualization, Data curation, Formal analysis, Supervision, Validation, Writing – original draft, Writing – review & editing. SD: Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing. MS: Conceptualization, Project administration, Supervision, Visualization, Writing – review & editing. MB: Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing. MV: Conceptualization, Validation, Writing – review & editing. ADj: Conceptualization, Supervision, Validation, Writing – review & editing. VeV: Conceptualization, Supervision, Validation, Writing – review & editing. VeI: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Francesca Spada,
European Institute of Oncology (IEO), Italy

REVIEWED BY
Donato Iacovazzo,
Barts Health NHS Trust, United Kingdom
Takao Ando,
Nagasaki University Hospital, Japan

*CORRESPONDENCE
Delmar Muniz Lourenço Jr
delmarmuniz@usp.br

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Case report: Insulinomatosis: description of four sporadic cases and review of the literature

Delmar Muniz Lourenço Jr[1,2*], Maria Lucia Corrêa-Giannella[3], Sheila Aparecida Coelho Siqueira[4], Marcia Nery[5], Flavio Galvão Ribeiro[1], Elizangela Pereira de Souza Quedas[1], Manoel de Souza Rocha[6], Ramon Marcelino do Nascimento[1] and Maria Adelaide Albergaria Pereira[5]

[1]Unidade de Endocrinologia Genética (LIM-25), Hospital das Clínicas (HCFMUSP), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil, [2]Instituto do Câncer do Estado de São Paulo (ICESP), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil, [3]Laboratório de Carboidratos e Radioimunoensaio (LIM-18), Hospital das Clínicas (HCFMUSP), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil, [4]Divisão de Patologia, Hospital das Clínicas (HCFMUSP), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil, [5]Divisão de Endocrinologia e Metabologia, Hospital das Clínicas (HCFMUSP), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil, [6]Departamento de Radiologia, Hospital das Clínicas (HCFMUSP), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil

The best-known etiologies of hyperinsulinemic hypoglycemia are insulinoma, non-insulinoma pancreatogenous hypoglycemic syndrome, autoimmune processes, and factitious hypoglycemia. In 2009, a disease not associated with classic genetic syndromes and characterized by the presence of multiple pancreatic lesions was described and named insulinomatosis. We present the clinical and pathologic features of four patients with the diagnosis of insulinomatosis, aggregated new clinical data, reviewed extensively the literature, and illustrated the nature and evolution of this recently recognized disease. One of our patients had isolated (without fasting hypoglycemia) postprandial hypoglycemia, an occurrence not previously reported in the literature. Furthermore, we reported the second case presenting malignant disease. All of them had persistent/recurrent hypoglycemia after the first surgery even with pathology confirming the presence of a positive insulin neuroendocrine tumor. In the literature review, 27 sporadic insulinomatosis cases were compiled. All of them had episodes of fasting hypoglycemia except one of our patients. Only two patients had malignant disease, and one of them was from our series. The suspicion of insulinomatosis can be raised before surgery in patients without genetic syndromes, with multiple tumors in the topographic investigation and in those who had persistent or recurrent hypoglycemia after surgical removal of one or more tumors. The definitive diagnosis is established by histology and immunohistochemistry and requires examination of the "macroscopically normal pancreas." Our case series reinforces the marked predominance in women, the high frequency of recurrent hypoglycemia, and consequently, a definitive poor response to the usual surgical treatment.

KEYWORDS

hyperinsulinemic hypoglycemia, insulinoma, insulinomatosis, *MAFA* gene, long-term outcome, postprandial hypoglycemia

1 Introduction

Spontaneous hypoglycemia in non-diabetic subjects can be occasionally difficult to assess. The initial approach should establish whether hypoglycemia is associated with excessive and inappropriate insulin production. Hyperinsulinemic hypoglycemia (HH) may be caused by insulinomas, non-insulinoma pancreatogenous hypoglycemic syndrome (NIPHS) that can be idiopathic or secondary to bariatric surgery, autoimmune processes (anti-insulin or anti-insulin receptor antibodies), and surreptitious administration of insulin or oral hypoglycemic agents (factitious hypoglycemia) (1–3).

The main etiology of HH is insulinoma, a neuroendocrine tumor originating from pancreatic beta cells that is usually small (1 to 1.5 cm), benign, and unique (4, 5). Due to these characteristics, its excision results in a permanent cure, and the recurrence of hypoglycemia is practically non-existent (5). In approximately 10% to 15% of cases, insulinoma is a malignant tumor, and thus, hypoglycemia can recur due to metastatic tumor disease (2, 5). Insulinomas may occur as part of multiple endocrine neoplasia type 1 (MEN1) syndrome and, more rarely, as part of von Hippel Lindau syndrome (VHL) or neurofibromatosis type 1 (NF1). In these contexts, pancreatic tumors are usually multiple, and the recurrence of tumors is common (6–8).

Insulinomatosis, a disease described in 2009 by Anlauf et al. (8), is characterized by the presence of multiple pancreatic lesions, both tumors and/or pretumor lesions, and it is not associated with classic genetic syndromes, such as MEN1, VHL, or NF1. Recurrence of hypoglycemia after surgery is frequent and probably results from the growth of smaller tumors or the development of new tumors from preexisting microlesions (8). In the present article, we report four cases of sporadic insulinomatosis and a review of the literature on this topic.

2 Methods

The laboratory diagnosis of HH was made by concomitant determination of the plasma glucose and serum insulin, C-peptide, and proinsulin during an episode of spontaneous hypoglycemia or provoked by prolonged fasting. During the fasting test, capillary measurements of blood glucose and ketonemia were performed every 30 to 60 min. The fast was interrupted when the patient had symptoms of hypoglycemia and capillary glucose ≤50 mg/dL or, if asymptomatic, when capillary glucose was between 45 and 50 mg/dL; it was also interrupted when capillary β-hydroxybutyrate concentrations were >1 mmol/L, as high values are indicative of hypoinsulinemia and make further fasting unnecessary (1, 5). A mixed meal test (MMT) was performed when patients had symptoms in the postprandial period; in this case, blood was collected for the determination of glucose and insulin, before and every 30 min for 5 h after the ingestion of a 400-calorie meal (64% of carbohydrates). The biochemical criteria for the diagnosis of HH were those recommended by the Endocrine Society (glucose ≤ 55 mg/dL, insulin ≥ 3 μUI/mL, C-peptide ≥ 0.6 ng/dL, proinsulin ≥ 5 pmol/mL, and negative sulfonylurea screen) (1). A β-hydroxybutyrate concentration ≤0.3 mmol/L was considered for the diagnosis of HH, as previously described in our series of insulinomas (5). For the topographic diagnosis of the tumor(s), pancreatic computed tomography (CT) or magnetic resonance imaging (MRI) and endoscopic ultrasonography (EUS) of the pancreas were used. In one case, we used ^{68}Gallium-DOTATATE PET-CT scanning to investigate the primary tumor and, in another, for recurrent hypoglycemia. Intra-arterial calcium stimulation with hepatic venous sampling (ASVS) for localization of insulinoma was performed when radiological methods were not able to identify the tumor (1, 5, 9).

Glucose was determined by the hexokinase method, insulin and C-peptide by chemiluminometric assays, and proinsulin by an immunoassay. Capillary ketonemia was assessed by the determination of blood β-hydroxybutyrate using an electrochemical method (MedSense Optium Meter). Blood sulfonylurea screening was performed by high-performance liquid chromatography (HPLC)/tandem mass spectrometry (Quest Diagnostics Nichols Institute, San Juan Capistrano, California, United States).

Sanger sequencing was provided to investigate germline mutations covering the coding area and splicing sites of the *MEN1* and *MAFA* genes. The protocol for the sequencing of the *MEN1* gene was conducted as previously reported (10, 11). Amplicons of exon 1 of the *MAFA* gene were amplified by polymerase chain reaction (PCR). PCR was performed in a total volume of 25 μL containing 2 μL of genomic DNA (200 ng), nuclease-free water 6 μL, 12.5 μL of Go Taq Green Master Mix (Promega, São Paulo, Brazil), primer forward 1.0 μL, primer reverse 1.0 μL, and DMSO 2.5 μL. The PCR thermocycling conditions were as follows: 10 min at 95°C, followed by 35 cycles of 40 min at 95°C, 40 min at 55°C as annealing temperature, followed by 40 min at 72°C and 10 min of final extension at 72°C.

The classification of genetic variants was conducted following the recommendations of the American College of Medical Genetics and Genomics and the Association for Molecular Pathologists (ACMG-AMP) (12).

3 Case presentations

3.1 Case 1

A 52-year-old woman was admitted with a 1-year history of recurrent episodes of palpitations, sweating, and dizziness. She had no family history of hypoglycemia or diabetes. During one of these episodes, the laboratory workout showed glucose of 37 mg/dL, insulin of 13.4 μUI/mL, C-peptide of 2.7 ng/dL, negative ketonemia, and a negative sulfonylurea screen (Table 1). Abdominal MRI and EUS did not identify tumors, but ASVS revealed a 6.2 insulin gradient in the splenic artery, consistent with higher insulin production in the pancreatic body/tail. A second abdominal MRI showed a 1-cm T2-hyperintense lesion, previously unnoted in the pancreatic tail, which was enucleated, and the histological/immunohistochemical diagnosis was insulin-positive well-differentiated neuroendocrine tumor (Ki67 < 3%). There was no remission of the hypoglycemic episodes, and a second fasting test confirmed HH. An abdominal MRI, conducted after 3 months,

TABLE 1 Clinical and biochemical patterns of the four Brazilian cases with sporadic insulinomatosis.

Clinical and biochemical findings											
Case	Sex	Age[a]	Age at the last medical care	Follow-up time (years)	Fasting hypoglycemia	Symptoms	History of symptoms up to the hypoglycemia diagnosis (years)	Fasting test Biochemical/hormonal data[b]	Malignancy	Longer hypoglycemia-free time (months)	Recurrent hypoglycemia after last medical care
1	F	52	54	2	Yes	Palpitations Sweating Dizziness	1	Glucose = 37 mg/dL Insulin = 13.4 µUI/mL C-peptide = 2.7 ng/dL Ketonemia = negative SU = negative	No	18	No
2	F	49	61	12	No[c]	Sweating Palpitations Tremors (after meals or exercise) 4 kg weight gain	4	PFT (65 h) = negative MMT (60 min) = positive (Figure 1)	No	132	No
3	F	40	43	3	Yes	Disorientation Mental confusion Convulsive episodes during the dawn (2)	3 (months)	PFT (14 h) = positive Glucose = 42 mg/dL Insulin = 8.1 µUI/mL Proinsulin = 12.4 pmol/L C-peptide = 2.25 ng/dl β-HB < 0.1 mmol/L SU = negative	No	15	Yes
4	F	22	47	25	Yes	Sweating Palpitations Feelings of faintness (after periods of prolonged fasting) Tonic–clonic seizures (2)	1	Glucose = 26 mg/dL Insulin = 15 µUI/mL β-HB < 0.1 nmol/L (Figure 2)	Yes	60	Yes

PFT, prolonged fasting test; SU, sulfonylurea screen; MMT, mixed meal test; β-HB, β-hydroxybutyrate.
[a]Age at admission.
[b]Exams at admission.
[c]Postprandial hypoglycemia.

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showed two pancreatic tail lesions of 1.3 and 0.9 cm, the latter being located near the site of the previously enucleated tumor. The sequencing of the *MEN1* and *MAFA* genes was provided, but no mutation detected. The following variants, found in homozygosis and classified as benign by ACMG, were identified in MAFA gene: c.582T>C (p.HIS194=) (rs1872900), a synonymous missense variant and; c.221_223del (p.HIS208del (rs141816779), an in-frame variant. Distal pancreatectomy was performed, and histopathological examination revealed two tumors measuring 0.6 and 1.1 cm with several smaller ones (<0.5 cm) along the pancreatic body; all of them were well-differentiated tumors (Ki67 < 3%) with positive immunohistochemistry staining for insulin, chromogranin A, and synaptophysin and negative staining for glucagon, confirming the diagnosis of insulinomatosis (Figure 1). The patient remains free of hypoglycemia for 18 months after the second surgery (Table 1).

3.2 Case 2

A 49-year-old woman was admitted with a 4-year history of episodes of sweating, palpitations, and tremors after meals that were occasionally triggered by exercise. She did not present neuroglycopenic symptoms and gained 4 kg (Table 1). She had no family history of hypoglycemia or diabetes. After 65 h, a prolonged fasting test was interrupted when a β-hydroxybutyrate of 1.1 mmol/L was detected, concomitantly with glucose of 72 mg/dL, insulin of 2.5 µUI/mL, proinsulin of 28.8 pmol/L, and C-peptide of 1.5 ng/dL. An MMT showed HH at 60 min (glucose = 38 mg/dL and insulin = 284.5 µUI/mL) (Figure 2A; Table 1). An abdominal MRI detected a 1.5-cm tumor in the tail of the pancreas, and a pancreatic EUS showed a 1.4-cm hypoechoic nodule in the same location (Figure 2A). The patient underwent enucleation of the pancreatic tumor, and the histological diagnosis was a pancreatic neuroendocrine tumor with positive immunohistochemistry for insulin, chromogranin, and synaptophysin and a Ki67 labeling index <3% (grade 1 tumor) (Figure 2A). Eight months after the surgery, hypoglycemia recurred, and a second prolonged fasting test was performed; the test was interrupted after 55 h when hyperketonemia was detected (1.1 mmol/L) concomitantly with a glucose of 68 mg/dL, insulin of <2.5 µUI/mL, and C-peptide of 1 ng/dL (Figure 2B). An MMT showed reactive hypoglycemia at 60 min (glucose = 55 mg/dL and insulin = 54 µUI/mL) (Figure 2B). The sequencing of *MEN1* and *MAFA* genes did not show mutations.This patient harbored the same MAFA benign

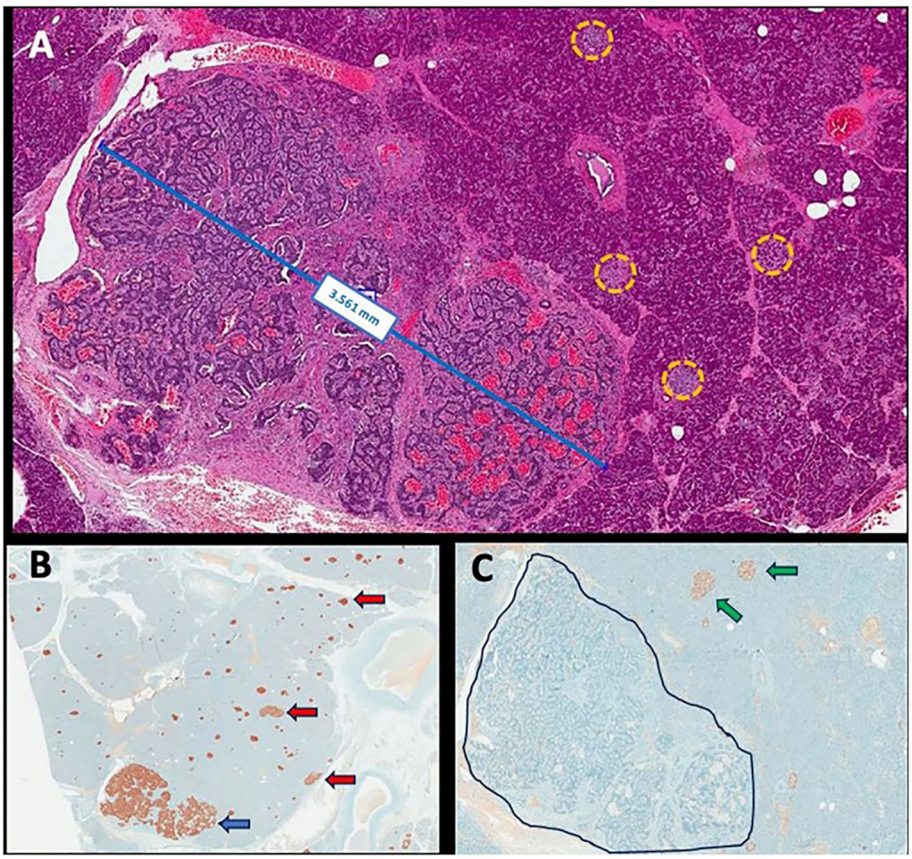
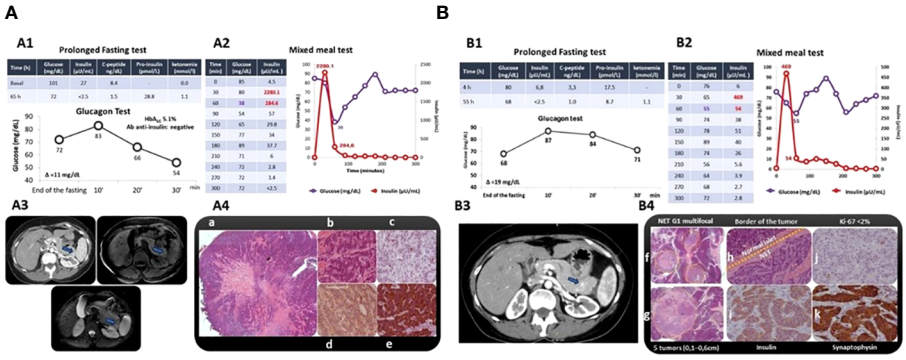


FIGURE 1
Representative histopathological images: neuroendocrine microtumor measuring 3.561 mm with several smaller clusters of neuroendocrine cells scattered within the macroscopically normal pancreas (yellow circles) (hematoxylin and eosin, ×200 magnification) **(A)**; immunohistochemistry for insulin showing positivity in neuroendocrine microtumor (blue arrow) and in the diverse smaller clusters of neuroendocrine cells (red arrow) (×100 magnification) **(B)**; immunohistochemistry for glucagon showing positivity in normal islets (green arrow) and negativity in the microtumor (delimited area) as in the clusters of neuroendocrine cells previously tested positive to insulin (×200 magnification) **(C)**.



Biochemical and hormonal tests, radiological features, and pathological findings at the first surgery (enucleation).

Biochemical and hormonal tests, radiological features and, pathological findings at the hypoglycemia recurrence eight months after when distal pancreatectomy was provided.

FIGURE 2
Patient with sporadic insulinomatosis (case 2) clinically presenting postprandial hyperinsulinemic hypoglycemia diagnosed for mixed meal test after normal response to prolonged fasting test. The same pattern of postprandial hypoglycemia was documented at the recurrence. Localization radiological exams and pathology documenting insulinomatosis after the first and second surgery are shown. **(A)** Biochemical and hormonal tests, radiological features, and pathological findings of the first surgery (enucleation). **(B)** Biochemical and hormonal tests, radiological features, and pathological findings of hypoglycemia recurrence 8 months after when distal pancreatectomy was provided. A1/B1. Prolonged fasting test was interrupted by the presence of ketonemia and the absence of hypoglycemia after 65 and 55 h with negative response at the glucagon test (increment <25 mg/dL); A2/B2. Mixed meal test was positive for hyperinsulinemic hypoglycemia 60 min on the first and second surgery; A3/B3. Pancreatic nodules were localized by computed tomography (A3/B3, arrow) and confirmed by endoscopic ultrasound (hypoechoic nodule measuring 1.5 cm in the pancreas tail (A3) and nodule in the distal extremity (1 cm) and hypervascularized nodule in the proximal segment of the pancreatic tail (0.6cm, arrow) (B3) whose biopsy was compatible with neuroendocrine neoplasia (IHC: insulin-positive); A4. Histology of the pancreatic nodule smaller (a) and larger in size (b) (hematoxylin and eosin); IHC documenting KI67 < 2 (c), positivity to chromogranin (d), and insulin (e); B4. Histology documenting three smaller pancreatic microtumors (f) and one larger tumor (g) stained by hematoxylin and eosin; transition between normal pancreatic tissue and neoplasia (h) (hematoxylin and eosin); IHQ revealing insulin-positive (i) well-differentiated neuroendocrine tumor (KI67 < 2%) (j), confirmed by positive IHC for synaptophysin (k).

variants identified, in homozygosis, in case 1: c.582T>C (p.HIS194=) (rs1872900) and c.221_223del (p.HIS208del (rs141816779). An abdominal CT revealed two hypervascularized nodules in the pancreas, one of them measuring 1 cm at the extremity of the pancreatic tail and another measuring 0.6 cm between the tail and the body; the same lesions were identified in the EUS (Figure 2B). The patient underwent distal pancreatectomy that allowed the diagnosis of five neuroendocrine tumors measuring up to 0.6 cm (Ki67 < 3%) compatible with insulinomatosis (Figure 2B). She remains free of hypoglycemia to date, 11 years after the last surgery.

3.3 Case 3

A 40-year-old woman was admitted with a history of disorientation, mental confusion, and two convulsive episodes that occurred during the dawn. She had no family history of hypoglycemia or diabetes. A prolonged fasting test of 14 h showed glucose of 42 mg/dL, insulin of 8.1 µUI/mL, proinsulin of 12.4 pmol/L, C-peptide of 2.25 ng/dl, β-hydroxybutyrate of <0.1 mmol/L, and a negative sulfonylurea screen (Table 1). A pancreatic MRI and an EUS did not identify tumors, but a ^{68}Gallium-DOTATATE PET-CT scanning revealed a focal area of uptake [standardized uptake value (SUV) of 7.1] in the head of the pancreas. Considering that the uptake could be physiological, the patient was submitted to an ASVS that, due to technical problems, was not able to localize the source of excessive insulin secretion. She received somatostatin analog monthly (30 mg) for 7 months with partial and insufficient improvement of hypoglycemia. Thus, the patient was referred to an exploratory laparotomy with intraoperative palpation and ultrasonography, which identified two lesions, one 0.6 cm in the body of the pancreas and another smaller one in the pancreatic tail; central pancreatectomy and resection of the distal nodule were performed, and the histological analysis revealed multiple well-differentiated neuroendocrine microtumors (Ki67 < 3%) measuring up to 0.5 cm compatible with insulinomatosis. The patient remained free of hypoglycemia for 15 months when tests performed after 12 h of fasting showed glucose of 50 mg/dL, insulin of 12.2 µUI/mL, and C-peptide of 3.64 ng/dL. She is now undergoing radiological examination for the topographical diagnosis. There was no mutation found in *MEN1* and *MAFA* genes. This patient harbored the same *MAFA* benign variants identified, in homozygosis, in cases 1 and 2: c.582T>C (p.HIS194=) (rs1872900) and c.221_223del (p.HIS208del (rs141816779).

3.4 Case 4

A 22-year-old woman was admitted with a 1-year history of episodes of sweating, palpitations, and feelings of faintness after periods of prolonged fasting; on two occasions, she reported tonic –clonic seizures. She had no family history of hypoglycemia or diabetes. During one of the episodes, the following measurements were recorded: glucose of 26 mg/dL and insulin of 15 µUI/mL (Table 1). An abdominal MRI showed a 1.4-cm tumor between the

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	1999*	2001	2004*	2005*	2008*	2009*	2016	2019**	2021**	2021	2023
Glucose (mg/dL)	26	80	45	37	40	38	70	45	15	119	75
C-peptide ng/dL	_	_	2.3	2.5	3.6	6.3	2.6	3.6	3.69	1.32	
Insulin (µU/mL)	15	7	14.9	14	6.9	1.5	14.6	26.4	29.6	7.9	9

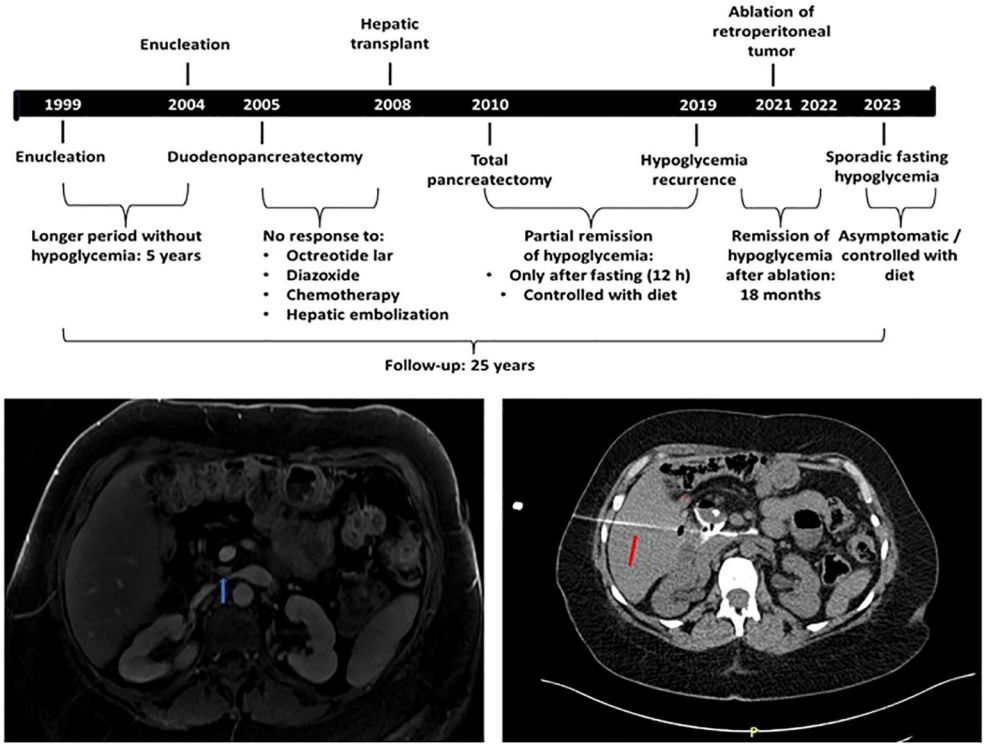

FIGURE 3
Long-term outcome (25 years) of a patient (case 4) diagnosed with malignant insulinomatosis 7 years after multiple surgical treatments by recurrent/persistent hypoglycemia. *, basal biochemical/hormonal values documenting hyperinsulinemic hypoglycemia before surgical procedures: 1999, enucleation; 2004, enucleation; 2005; subtotal duodenopancreatectomy; 2008, liver transplant after biopsy confirming hepatic metastasis and unsuccessful multiple treatments (diazoxide, somatostatin analogs, chemotherapy, and hepatic embolization); 2019/2021, basal biochemical/hormonal values documenting hyperinsulinemic hypoglycemia before radiofrequency ablation (red arrow: needle of ablation) of retroperitoneal tumoral mass (blue arrow) detected by magnetic resonance imaging in 2021.

head and body of the pancreas. The patient was referred for tumor enucleation, and histological examination showed a grade 1 pancreatic neuroendocrine tumor with positive immunohistochemistry for chromogranin and insulin and a Ki67 labeling index <3% (1999). The patient remained asymptomatic and had normal biochemical and hormonal findings for 5 years, after which she presented recurrence of hypoglycemia. An HH was redocumented, MRI topographic exploration was negative, and an ASVS showed hypersecretion of insulin from the head of the pancreas. The patient was referred for a second surgery which allowed the identification and enucleation of a small tumor (0.5 cm), whose pathology revealed a neuroendocrine tumor with a Ki67 <5%, but remission of hypoglycemia was not achieved (2004). Again, topographic investigation remained negative by MRI and positive by ASVS at the same localization. With this, pancreatoduodenectomy was performed (2005). The histological diagnosis was compatible with insulinomatosis by the presence of multiple neuroendocrine tumors (up to 0.7 cm), with Ki67 between 2% and 10%. Also, the sequencing of *MEN1* and *MAFA* genes did not reveal any mutations. This patient harbored the same *MAFA* benign variants identified, in homozygosis, in cases 1, 2 and 3: c.582T>C (p.HIS194=) (rs1872900) and c.221_223del (p.HIS208del (rs141816779). Due to persistent hypoglycemia, a new radiological exam was done (^{111}In-Octreotide scintigraphy and abdominal MRI) and suggested hepatic metastasis. A hepatic biopsy confirmed the diagnosis of metastatic neuroendocrine neoplasia with positive immunohistochemistry for insulin (2005). Clinical treatment (diazoxide and octreotide) was initiated with partial and transient improvement of hypoglycemia. Hepatic artery embolization and systemic chemotherapy were performed without hypoglycemia remission. Between 32 and 34 years of age, as the patient had metastasis only in the liver and, probably, a persistent pancreatic disease, she was referred for a liver transplant (2008) with transient remission of hypoglycemia and then underwent total pancreatectomy (2010); histopathological and immunohistochemistry examination revealed metastatic neuroendocrine neoplasia and insulinomatosis,

respectively. Transient improvement of hypoglycemia after total pancreatectomy, without development of diabetes, was followed by gradual and progressive worsening requiring fractionated diet in subsequent years (2011–2020), but abdominal MRI exams performed periodically and, sometimes, complemented with ^{111}In-Octreotide scintigraphy or ^{68}Gallium-DOTATATE PET-CT, were persistently negative. Between 2005 and 2021, the somatostatin analog was offered to the patient for short periods of less than 6 months immediately after failure to remit hypoglycemia for different treatments such as liver embolization, liver transplant, or total pancreatectomy. However, intolerance or unsatisfactory adherence prevented an accurate assessment of the potential of this drug to correct hypoglycemia in this patient. At 45 years old, after an episode of neuroglycopenia (glucose of 15 mg/dL, insulin of 29.6 μUI/mL, C-peptide of 3.69 ng/dL, and β-hydroxybutyrate of <0.1 mmol/L), an abdominal MRI identified a small suspect lesion near the site where it would be the head of the pancreas, whereas a ^{68}Gallium-DOTATATE PET-CT scan was inconclusive (2021). The patient was referred for radiofrequency ablation guided by CT. After 1 h, glucose measurements were approximately 150 and 200 mg/dL, and during the next 6 months, she required exogenous insulin for treating secondary diabetes mellitus. Since then, she began to experience new episodes of fasting hypoglycemia remitting after a meal, which led to the suspension of exogenous insulin. The pre- and postprandial plasma glucose values are approximately 90 and 160 mg/dL, respectively (Figure 3).

4 Discussion

Insulinomatosis is a pancreatic disease characterized by multiple micro- and macrotumors and foci of beta-cell hyperplasia (8, 13–15). Although there were previous reports of "multiple" insulinomas (13), the pathological bases that allowed its differentiation from insulinoma were only defined in 2009 by Anlauf et al., who evaluated the histological and immunohistochemical characteristics of the tumors of the pancreas in 253 patients with sporadic single insulinoma, 13 patients with insulinoma associated with MEN1, and 14 patients (mean age of 41 ± 12 years old; 10 women and 4 men) with what these authors called insulinomatosis (8). In this condition, the histological evaluation of the pancreas revealed several insulinomas [microtumors (<5 mm) and macrotumors (≥5 mm)] and multiple conglomerates of beta cells of variable size called insulin-expressing monohormonal endocrine cell clusters (IMECCs). Immunohistochemistry of tumors and IMECCs was positive for insulin and negative for glucagon. IMECCs were initially considered precursor lesions of micro- and macrotumors, and Anlauf et al. highlighted that the presence of these pretumoral lesions only occurred in insulinomatosis and never in sporadic insulinomas or associated with MEN1. Recently, the term IMECC was incorporated as microtumors (<0.5 cm). Immunohistochemistry in sporadic benign insulinoma, which in the authors' experience was always a single tumor, was positive for insulin and negative for glucagon. In the tumors of patients with MEN1, immunohistochemistry was variable in the different tumors and could be negative or positive for insulin, glucagon, or pancreatic polypeptide (PP). Thus, in this genetic syndrome, tumors may be non-functioning, or they may express one of the islet hormones. It was notable that patients with insulinomatosis showed a high rate of HH persistence/recurrence despite repeated surgeries. Insulinomatosis was a benign disease in all patients except one who presented with metastatic disease (8). After this description, others emerged describing new cases, all highlighting that, in insulinomatosis, the recurrence rate of hypoglycemia after surgery is high (16–19), contrasting with the absence of recurrent hypoglycemia in sporadic well-differentiated insulinomas (8) and with a low recurrence rate of hypoglycemia, even with high rates of tumoral recurrence, in cases with MEN1-related insulinoma undergoing pancreatic surgery (~7%) (6–8, 20, 21).

We described four adult women presenting HH with histological and immunohistochemical diagnoses of insulinomatosis. All of them had biochemical persistence or recurrence of hypoglycemia after the initial pancreatic surgery. One of the patients (case 1) had persistent hypoglycemia, while the second (case 2) had recurrent hypoglycemia 8 months after enucleation of what was thought to be sporadic insulinomas, and they were subsequently submitted to partial pancreatectomy, which allowed the histological diagnosis of insulinomatosis. They remained free of hypoglycemia 1.5 and 11 years, respectively, after the last procedure. Case 3 had persistent HH after partial pancreatectomy, which allowed the diagnosis of insulinomatosis; this patient had recurrent HH 15 months after the last surgery. In all these three cases, the return of hypoglycemia after surgery is linked to the recurrence of pancreatic disease (micro- and macrotumors). In case 4, the only one with a diagnosis of metastatic malignant insulinomatosis, recurrences of hypoglycemia over a period of 25 years of follow-up were due to the growth of new tumors in the pancreas, but also due to extrapancreatic (metastatic) disease. It is interesting to highlight that this is the only patient in the literature who underwent a liver transplant and subsequent radiofrequency ablation of the residual retroperitoneal lesion (Figure 3).

Overall, there are 27 cases documented with sporadic insulinomatosis including our four cases presently reported (8, 17, 22–25) (Table 2). Of note, one of these 27 cases previously reported had proinsulinomatosis, and this diagnosis was probable in another sporadic case with undetectable insulin and high levels of proinsulin (8, 21, 23). The marked predominance in women is reinforced by the present cases (81%; 22/27). The mean age at the diagnosis of hypoglycemia in these sporadic cases was 42 ± 13.4 years (17–64) with 41% of them (11/27) undergoing two or more surgical resections (2 ± 1.06; 1–5) for recurrent hypoglycemia (Table 2). All of our cases had at least one recurrence and three of them underwent more than one surgery. The recurrence was pancreatic and due to benign insulinomatosis in three of them, while case 4 had recurrence secondary to pancreatic insulinomatosis and to metastatic insulinomatosis. This is the second case with sporadic malignant insulinomatosis reported so far (7.4%; 2/27) (Tables 1, 2). The first one, reported by Anluf et al. (8), underwent wedge resection for hepatic metastasis. This predominance of benign behavior is also observed in familial insulinomatosis, as malignancy was not present in any of the 12 cases of insulinomatosis from three families reported so far (8, 13, 14, 22, 26).

It is not possible to differentiate between insulinoma and insulinomatosis before surgery. Our previous experience with insulinoma (5) allows us to make some comparisons between

TABLE 2 Clinical data of all 27 cases with sporadic insulinomatosis reported by literature, including four patients of the present study.

Reference	Case	Sex	Age[a]	Recurrent hypoglycemia after first surgery	Number of surgeries	Biochemical/ hormonal data	Fasting hypoglycemia	Malignancy	Proinsulinomatosis	Other oncologic treatments	^{68}Ga-DOTATATE PET/CT	First surgery	Last surgery
Anluf (2009) (8)	1	F	17	Yes	2	NA	Yes	No	No		–	PP	Enucleation
	2	F	26	No	1	NA	Yes	No	No		–	PP	–
	3	F	28	Yes	3	NA	Yes	No	No		–	Enucleation	PP
	4	F	32	Yes	2	NA	Yes	No	No		–	Enucleation	PP + enucleation
	5	F	37	No	1	NA	Yes	No	No		–	PP	–
	6	F	40	Yes	5	NA	Yes	Yes	No		–	Enucleation	Lymphadenectomy + Wedge resection (liver)
	7	F	43	No	1	NA	Yes	No	No		–	PP	–
	8	F	45	No	1	NA	Yes	No	No		–	PP	–
	9	F	49	No	1	NA	Yes	No	No		–	PP	–
	10	M	50	No	1	NA	Yes	No	No		–	PP	–
	11	M	57	No	1	NA	Yes	No	No		–	PP	No
	12	M	56	Yes	1	NA	Yes	No	No		–	PP	– (autopsy)
	13	M	59	NA	1	NA	Yes	No	No		–	PP	–
Iacovazzo (22)[c]	14	F	17	Yes	3	Yes	Yes	No	No		–	Enucleation	Whipple
	15	F	48	Yes	1	Yes	Yes	No	No	Octreotide[b]	–	PP	–
	16	F	64	Yes	2	Yes	Yes	No	Yes		–	PP	PP
	17	F	47	No	1	Yes	Yes	No	No		–	PP	–
	18	F	51	No	1	Yes	Yes	No	No		–	PP	–
	19	F	20	NA	1	No	Yes	No	No		–	PP	–
Snaith (2020)[d] (17)[d]	20	F	40	Yes	3	Yes	Yes	No	No	Octreotide[b] Everolimus[b]	Negative[e]	Enucleation	TP
Mintziras (2021)[d] (23)	21	F	48	Yes	2	Yes	Yes	No	Yes		Positive	Enucleation	PP
Anoshkin (2021) (24)	22	M	60	No	1	No	Yes	No	No		Negative[e]	PP	–

(Continued)

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TABLE 2 Continued

Reference	Case	Sex	Age[a]	Recurrent hypoglycemia after first surgery	Number of surgeries	Biochemical/hormonal data	Fasting hypoglycemia	Malignancy	Proinsulinomatosis	Other oncologic treatments	^{68}Ga-DOTATATE PET/CT	First surgery	Last surgery
Tartaglia (2022) (25)	23	F	41	Yes	1	Yes	Yes	No	No	Octreotide[f]	Positive	PP	–
Lourenço (2023)[d]	24	F	52	Yes	2	Yes	Yes	No	No		–	Enucleation	PP
	25	F	49	Yes	2	Yes	No[g]	No	No		–	Enucleation	PP
	26	F	40	Yes	1	Yes	Yes	No	No	Octreotide[b]	Inconclusive	PP	–
	27	F	22	Yes	6	Yes	Yes	Yes	No	Octreotide[b]	Inconclusive[e]	Enucleation	TP Hepatic transplant RFA

PP, partial pancreatectomy; TP, total pancreatectomy; RFA, radiofrequency ablation.
[a]Age at diagnosis.
[b]Poor or partial responses or intolerance to somatostatin analogs or everolimus.
[c]Nine sporadic cases were genetically investigated by Iacovazzo et al. (three of them had been clinically reported by Anluf et al. (8), and they were negative for germline and somatic MAFA mutations.
[d]Six additional cases whose genetic testing was negative for germline MAFA mutations.
[e]Performed during follow-up in one of the episodes of hypoglycemia recurrence after the first surgery.
[f]Highly responsive to a somatostatin analog.
[g]Positive mixed meal test.
NA, not available.

isolated insulinoma and insulinomatosis. All patients with insulinomatosis were women, while an even sex distribution was observed in patients with insulinoma; however, although the absolute prevalence of insulinomatosis in women should be emphasized, this does not allow the differentiation between the two conditions (1, 5, 8). The clinical picture is not different in patients with insulinomatosis and insulinoma, but we would like to point out that one of the four patients with insulinomatosis had hypoglycemia only in the postprandial period, with no fasting hypoglycemia, while this did not occur in any of our patients with isolated insulinoma (5). From our knowledge, this is the first case with insulinomatosis presenting postprandial hypoglycemia documented with mixed meal test reported so far (Figure 2), contrasting with fasting hypoglycemia noticed in the other 26 sporadic (Table 2) (8, 17, 22–25) and in 11 familial cases with insulinomatosis (8, 13, 22, 26).

The biochemical evaluation is identical in insulinoma and insulinomatosis, and the findings of topographic investigation may also be similar, especially when only one tumor is identified. In insulinomatosis, the multiple tumors can be synchronous or asynchronous, and in the latter case, one tumor may appear before the other(s) develop. Failure to identify tumors by the usual radiological methods (magnetic resonance imaging, computed tomography), including ^{68}Gallium-DOTATATE PET-CT and even endoscopic ultrasound, is common as most tumors in insulinomatosis are too small (microtumors, <5 mm) to be detected by these imaging diagnostic techniques, making preoperative diagnosis of the disease difficult. This context should motivate the performance of an ASVS to localize the source of excessive insulin secretion to guide surgical removal (1). It is important to highlight that ASVS may have the same limitations as radiological methods due to the simultaneous and additive functionality of the multiple microtumors spread across the pancreas, without the identification of a dominant area responsible for hypersecretion of insulin. Only the identification of more than one territory as a source of insulin hypersecretion can raise the suspicion of insulinomatosis. Therefore, the presurgical diagnosis of this disease can only be considered when several tumors are identified or suspected by the usual radiological methods or by ASVS. When multiple tumors are identified, it is mandatory to exclude clinically and/or molecularly the diagnosis of MEN1 (6–8).

Thus, the definitive diagnosis of insulinomatosis is histological and requires examination of the peritumoral "macroscopically normal pancreas," not just the tumor. In the "normal" pancreas, micro- and macrotumors will be identified (8, 27, 28). If the surgical approach was tumor enucleation, a complete analysis of the pancreas is not feasible. In this case, the patient may remain symptomatic because there are other macro- or microtumors not identified by presurgical (MRI/CT or EUS) or intrasurgical methods. Hypoglycemia may also improve and recur later due to the growth of preexisting tumors or the development of new ones. The histological differential diagnosis between insulinomatosis and MEN1 is easily established when the criteria mentioned above are adopted (8, 18). In patients with MEN1, the occurrence of multiple neuroendocrine tumors is frequent, but in general, immunohistochemistry shows that they are quite diverse and

may be non-functioning or may produce insulin, glucagon, or pancreatic polypeptide. In insulinomatosis, micro- and/or macrotumors are present, and immunohistochemistry is quite monotonous and always positive only for insulin in the various existing lesions. Tumor recurrence occurs earlier in patients with insulinomatosis than in those with MEN1 (8). In our service, we did not have any recurrence of hypoglycemia in patients operated on for benign insulinoma or insulinoma with MEN1 (5).

Insulinomatosis is a disease that requires a difficult and, sometimes, exhausting surgical treatment. Due to the rarity of the disease, the treatment with somatostatin analogs has not yet been evaluated. In our series, one of our cases (case 3) had no response after a short period of drug use, while another did not tolerate the analog (case 4) (Table 2). Also, two cases with sporadic insulinomatosis and three with familial insulinomatosis received this treatment, and response/tolerance seemed to be unsatisfactory (14, 17, 22) (Table 2). However, one clinical finding that drew our attention was the good response to therapy with a somatostatin analog in a patient with suspected insulinomatosis. This diagnosis assumption was made because the patient had HH, two pancreatic tumors, and negative clinical and genetic findings for MEN1 (data not shown). She was not included in this series because she did not undergo surgery and, therefore, did not have a definitive histological diagnosis of insulinomatosis. This patient showed absolute remission of hypoglycemia and a decrease in tumor size after therapy with the analog. A similar response was recently described in a patient with insulinomatosis (25) (Table 2).

In this scenario, the role of somatostatin analogs as one of the options to control hypoglycemia and the growth of tumors in insulinomatosis cases requires additional studies to elucidate if it diverges with the poor response noticed with most of the isolated insulinomas (5). Anyway, a possible explanation for this potential difference could be that the production and secretion of insulin, as well as the growth of micro- and macrotumors, can be more easily controlled by beta-cell inhibitors than by an autonomous tumor. In this rationale, it is relevant to consider that there are only a few cases investigated with ^{68}Gallium-DOTATATE PET-CT, an exam that could predict response to somatostatin analogs (Table 2). In fact, only three sporadic cases, including case 3 of the present study, had ^{68}Gallium-DOTATATE PET-CT as an initial approach. This exam was negative in our case 3, who had no clinical response to the analog, while it was positive in that patient highly responsive to somatostatin analogs and in another with proinsulinomatosis (23, 25). Other five cases had ^{68}Gallium-DOTATATE PET-CT during the investigation of hypoglycemia recurrence (two familial, three sporadic), including our case 4, and in all of them, this exam was negative or inconclusive to localization of tumors (17, 22, 24). We consider that, in any case with this disease, we must attempt clinical treatment with the analog before performing partial or total pancreatectomy. In fact, total pancreatectomy is not always a definitive solution to hypoglycemia in insulinomatosis, as documented in our case 4 and as previously reported by Snaith et al. (17).

Insulinomatosis is generally sporadic, but familial cases with autosomal dominant inheritance have been described (8, 13, 22, 26). Although a family with patients affected by multiple insulinomas and hypoglycemia had been reported in 1977 (13), only in 2018 were germline mutations in the *MAFA* gene identified in two families in which members with hypoglycemia secondary to insulinomatosis coexisted with members diagnosed with diabetes mellitus (22). An important issue is how the same genetic alteration can lead to apparently opposite diseases (22). More recently, a novel missense *MAFA* mutation was reported in a kindred with familial insulinomatosis (26). Overall, a germline *MAFA* mutation has been documented in only three families to date and one of them was Brazilian (22, 26). Of note, our unrelated sporadic cases do not harbor the mutation p.Ser64Phe found in large Brazilian family with insulinomatosis previously reported (22). Overall, germline *MAFA* mutations were not found in our 4 cases and 11 other sporadic cases (17, 22, 23). By negative genetic analysis, the possibility of familial insulinomatosis as a result of a *de-novo* *MAFA* mutation or incomplete penetrance or even a founder mutation from apparently unrelated clusters was excluded in our cases.

It is noteworthy that the same in-frame deletion was found in all four currently reported cases with proven insulinomatosis and in a fifth case with presumed insulinomatosis (not yet operated on due to excellent response to somatostatin analogue). This variant, located in a region with ten successive histidine repeats, was previously documented in a case of proinsulinomatosis and its benign nature is reinforced by its very high frequency in genomic banks (>81% in genomes from gnomD) (14, 23).

In addition, in nine sporadic cases, somatic *MAFA* mutations were excluded (22). While such mutations have not been identified so far, we cannot entirely rule out, due to the rarity of the disease, whether they can play a role in the pathogenesis of sporadic insulinomatosis. Recently, new copy number variations in *ATRX*, *FOXL2*, *IRS2*, and *CEBPA* genes were documented in macro- and microtumors of one case with sporadic insulinomatosis, suggesting a potential role of these genes in the pathogenesis of this rare disorder (24).

5 Conclusions

The main considerations to be drawn from the analysis of our data and those of the literature are that the suspicion of insulinomatosis can be raised: 1) before surgery, in patients with multiple tumors in the topographic investigation (after MEN1 exclusion) or in whom more than one territory was identified as sources of insulin hypersecretion by ASVS; 2) after surgical removal of one or more tumors, in those patients who had no improvement or recurrence of the hypoglycemic condition; and 3) the definitive diagnosis is established by histology and immunohistochemistry and requires examination of the "macroscopically normal pancreas."

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by CAPPesq (no. 70989723.0.0000.0068). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

DL: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. MC-G: Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. SS: Data curation, Formal analysis, Methodology, Writing – review & editing. MN: Data curation, Formal analysis, Writing – review & editing. FR: Formal analysis, Investigation, Writing – review & editing. EQ: Formal analysis, Investigation, Methodology, Writing – review & editing. MR: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. RN: Data curation, Formal analysis, Investigation, Writing – review & editing. MP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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EDITED BY
Bojana Popovic,
University of Belgrade, Serbia

REVIEWED BY
Maria Vittoria Davi',
University of Verona, Italy
Marina Tsoli,
National and Kapodistrian University of Athens, Greece

*CORRESPONDENCE
Yegui Jia
Jyg218@sina.com

†These authors have contributed equally to this work and share first authorship

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Outcomes of endoscopic ultrasound-guided ablation and minimally invasive surgery in the treatment of pancreatic insulinoma: a systematic review and meta-analysis

Dan Xiao[1†], Li Zhu[2†], Si Xiong[3], Xu Yan[4], Qin Jiang[1], Ao Wang[1] and Yegui Jia[1*]

[1]Department of Gastroenterology, The Sixth Hospital of Wuhan, Affiliated Hospital of Jianghan University, Wuhan, Hubei, China, [2]Gastrointestinal Surgery, The Sixth Hospital of Wuhan, Affiliated Hospital of Jianghan University, Wuhan, Hubei, China, [3]Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (HUST), Wuhan, Hubei, China, [4]Internal Medicine Department, University Hospital, Wuhan Institute of Technology, Wuhan, Hubei, China

Background and aims: Most pancreatic insulinomas can be treated by minimally invasive modalities. The aim of this meta-analysis was to assess the clinical outcomes of endoscopic ultrasound (EUS)-guided ablation and minimally invasive surgery (MIS) in the treatment of pancreatic insulinoma.

Materials and methods: Online databases were searched for relevant studies. The primary aim was to compare the rates of adverse events (AEs) and the secondary aims were to compare the clinical and technical success rates, length of hospital stays, and symptom recurrence rates between EUS and MIS approaches.

Results: Eight studies with 150 patients were identified that reported EUS-guided ablation outcomes, forming the EUS group, and 9 studies with 236 patients reported MIS outcomes, forming the MIS group. The pooled median age of the included patients in the EUS group was greater than that of the MIS group (64.06 vs. 44.98 years old, $p < 0.001$). Also, the technical success rate was significantly higher in the EUS group (100% vs. 96.6%, $p = 0.025$), while the clinical success was significantly higher (6%) in the MIS group (94% vs. 98.7%, $p = 0.021$). The AE rates (18.7% vs. 31.1%, $p = 0.012$) and severe AE rates (1.3% vs. 7.9%, $p = 0.011$) were significantly lower in the EUS group. The median length of hospital stay in the EUS group (2.68 days, 95% CI: 1.88–3.48, $I^2 = 60.3\%$) was significantly shorter than in the MIS group (7.40 days, 95% CI: 6.22–8.58, $I^2 = 42.2\%$, $p < 0.001$). The recurrence rate was significantly higher in the EUS group (15.3% vs. 1.3%, $p < 0.001$).

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Conclusions: EUS-guided ablation is associated with a lower AE rate and a shorter length of hospital stay, but a higher recurrence rate for the treatment of insulinoma compared with MIS. The EUS approach may be an alternative, even first-line, treatment for poor surgery candidates.

KEYWORDS

insulinoma, endoscopic ultrasound, ablation, minimally invasive surgery, adverse event, clinical outcomes

1 Introduction

Pancreatic neuroendocrine tumors (panNETs) account for less than 2% of all pancreatic tumors (1). According to the presence or absence of a clinical hormonal hypersecretion syndrome, panNETs are classified into functional or non-functional tumors. The most prevalent functional panNET is insulinoma (2). Insulin hypersecretion and hypoglycemia, which are associated with hypoglycemic, neuroglycopenic, and sympathetic-overstimulation symptoms, are the main manifestations of insulinoma (1). The early occurrence of obvious clinical symptoms of insulinoma generally allows its early diagnosis, when the insulinoma will still be small, commonly ranging in size from 5 to 20 mm at the time of early diagnosis (3).

Most insulinomas are benign single tumors, and surgical resection is the main treatment modality (4). However, there is a considerable risk of adverse events (AEs) with pancreatic surgery. A systematic review with 62 studies indicated that postoperative pancreatic fistula, delayed gastric emptying (DGE), and hemorrhage occurred in 14%–58%, 5%–18%, and 1%–7% of panNET cases after surgery, respectively, and even in-hospital death in 3%–6% of patients (5).

Therefore, alternative therapy modalities with a less invasive nature have attracted increasing attention and some have been used in clinical settings. Minimally invasive surgery (MIS), including laparoscopic and robotic surgery for insulinomas, has been reported to be associated with a lower incidence of AEs, shorter hospital stays, and a similar treatment efficacy when compared with open surgery (6). Recently, endoscopic ultrasound (EUS)-guided ablation, including radiofrequency ablation (RFA) and ethanol ablation (EA), has been reported. Considering the generally small size and benign nature of insulinomas, the endoscopic approach may be an optimal alternative to surgical resection. However, there are scant studies comparing the outcomes of EUS-RFA, EUS-EA, and surgery, especially minimally invasive surgery.

To fill this gap, we conducted a systematic review and meta-analysis. The primary aim was to compare the rates of adverse events (AEs) and the secondary aims were to compare the clinical and technical success rates, length of hospital stays, and symptom recurrence rates between EUS and MIS approaches.

2 Materials and methods

2.1 Search strategy

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed for conducting this meta-analysis. Through systematic searches of the PubMed, Cochrane Library, and Web of Science databases, we were able to retrieve literature in English that had been published from the time the databases were created until December 1, 2023. We used the following Medical Subject Heading (MeSH) terms to search the literature in the aforementioned databases: "insulinoma," "endoscopic ultrasound," "radiofrequency ablation," "ethanol ablation," "minimally invasive surgery," "laparoscopic surgery," and "robotic surgery." Only articles in English were searched and checked.

2.2 Selection criteria

The study inclusion criteria were as follows (1): clinical studies with human patients; (2) patients diagnosed with insulinoma treated with EUS-guided ablation or MIS; and (3) studies where the AEs, clinical and technical success rates, length of hospital stays, and symptom recurrence rates were reported; (4) studies that were classed as medium and high quality according to the Newcastle–Ottawa scale (NOS).

The exclusion criteria were as follows: (1) editorials, letters, reviews, meta-analyses, protocols, and case reports; (2) no detailed results were provided or the outcomes were not clear; (3) insulinomas were contaminated with other panNETs; and (4) duplicate studies. Finally, a full-text check was conducted to examine whether the identified papers met the inclusion criteria and passed the exclusion criteria. Two independent researchers performed the above processes, and their search results were consistent.

2.3 Quality assessment

We used the NOS as an assessment indicator since most of the relevant research in the studies was retrospective or single-arm. Studies

with an NOS rating of 7–9 were considered high quality, while those with an NOS rating of 4–6 were considered medium quality.

2.4 Data extraction

Two independent researchers extracted the data from the included papers. If disagreements existed, they were resolved by the other co-authors. The following data were extracted: last name of the author, year of publication, study country, ages of the patients, number of patients with insulinoma, treatment methods, AEs, clinical and technical success rates, length of hospital stays, and symptom recurrence rates. A severe AE was defined as an AE that needed re-intervention, or had a Clavien-Dindo classification ≥ III.

Clinical success was defined as the recovery from insulinoma-associated symptoms. Symptom recurrence was defined as the recurrence of insulinoma-associated symptoms.

2.5 Statistical analysis

The primary aim was to compare the AEs between the EUS and MIS approaches. The secondary aims were to compare the clinical and technical success rates, length of hospital stays, and symptom recurrence rates between EUS and MIS approaches. The above endpoint proportions were pooled and analyzed. The I^2 value was used to assess heterogeneity between the studies. A random effect result was used with an otherwise fixed-effect outcome, with $I^2 > 50\%$ deemed significantly heterogeneous. Sensitivity analysis was used to find the potential study that could cause significant heterogeneity. Visual examination of the funnel plot and quantitative analysis utilizing Egger's test of the intercept were used to evaluate publication biases. All the statistical analyses were conducted with Stata (Version 14). A *p-value* of 0.05 was considered statistically significant.

3 Results

3.1 Search results

After searching the aforementioned databases, 63 studies were identified that reported EUS-guided ablation for insulinoma, and 84 studies reported MIS for insulinoma.

Among the EUS studies, 5 were duplicate studies, 23 studies were case reports or case series reports with a sample size ≤ 5, 18 studies were reviews and meta-analyses, 2 studies were irrelevant, and 7 studies had no clinical outcomes. Finally, 8 studies with 150 patients were included in the data analysis (Figure 1) (7–14).

Among the MIS studies, 5 were duplicate studies, 15 studies were case reports or case series reports with a sample size ≤ 5, 21 studies were reviews and meta-analyses, 2 studies were irrelevant, and 32 studies had no clear clinical outcomes for laparoscopic surgery. Finally, 9 studies with 236 patients were included in the data analysis (15–23).

All the studies were conducted in referral centers, and the main indications for patients choosing EUS-RFA were that they were not good candidates for surgery or were unwilling to undergo surgery.

3.2 Quality assessment

All 17 studies identified in the initial screening mentioned above were retrospective studies, and they all underwent a quality appraisal using the NOS system (Supplementary Table 1) by two independent authors. Among these, 11 studies were assessed as medium quality, and six studies were assessed as high quality, according to the NOS scale, and thus passed the quality criterion. Consequently, the 17 studies were all included in our meta-analysis. The patient characteristics and study endpoints of the included studies are presented in Tables 1, 2, respectively.

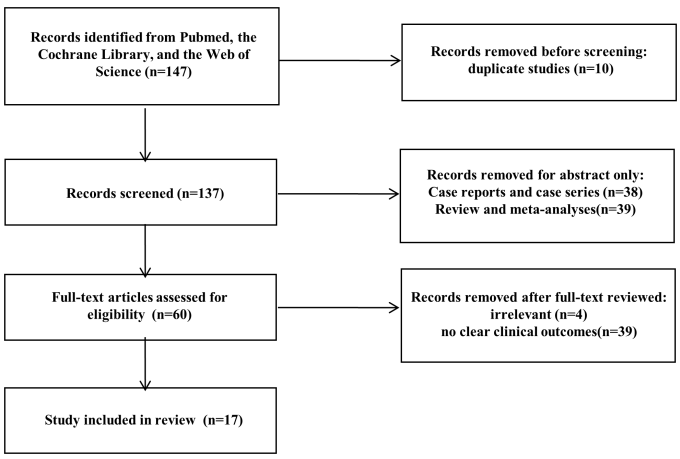

FIGURE 1
Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram showing the study selection process.

TABLE 1 Characteristics of the included studies.

Author	Publication year	Study design	Country	Total patients, n	Number of lesions, n	Treatment method	Median age (range), y	Median tumor size, mm (range)	Tumor location, (H/N/B/T)	Tumor grade, (G1/G2)
Debraine et al. (7)	2023	Retrospective	Belgium	11	11	RFA	65 (49–84)	11.6 (6.0-22.0)	5/1/3/2	9/2
Oleinikov et al. (8)	2019	Retrospective	Israel	7	9	RFA	60 (28-82)	13.0 (12.0-19.0)	7/0/2/0	9/0
Marx et al. (9)	2021	Retrospective	Switzerland	7	7	RFA	66 (48-97)	13.0 (8.0-20.0)	1/3/2/1	4/1
Sada et al. (10)	2023	Retrospective	US	8	8	EA	69.5 (34-84)	17.0 (12.1, 21.0)	4/1/1/2	NR
Yan et al. (11)	2022	Retrospective	China	9	10	EA	60 (32-69)	17.0 (11.0-21.0)	7/0/2/1	NR
Andreis et al. (12)	2023	Retrospective	Italy	10	10	RFA	65.5 (51-84)	11.0 (8.0-19.0)	3/0/3/4	9/1
Jürgensen et al. (13)	2023	Retrospective	Germany	9	9	EA	68 (57-79)	14.0 (7.0-21.0)	1/0/5/3	NR
Crinò et al. (14)	2023	Retrospective	Italy	89	89	RFA	55.0 (39-71)	13.0 (9.0-21.0)	34/0/39/16	66/3*
Espan˜a-Go´ mez et al. (15)	2009	Retrospective	Mexico	14	14	Laparoscopic	42 (28-56)	20.0 (11.0–52.0)	1/1/7/5	NR
Belfiori et al. (16)	2018	Retrospective	Italy	15	15	MIC-EN	39 (24–77)	12.5 (9–26)	4/0/5/6	NR
Cunha et al. (17)	2007	Retrospective	France	12	12	Laparoscopic	48 (29-67)	13.0 (7.5-18.5)	2/4/1/5	NR
Hu et al. (18)	2011	Retrospective	China	43	43	Laparoscopic	42 (27.5-56.5)	14.5 (8.0-21)	9/10/12/12	NR
Isla et al. (19)	2007	Retrospective	UK	21	21	Laparoscopic	46 (22–70)	NR	5/0/9/7	NR
Nakamura et al. (20)	2015	Retrospective	Japan	15	16	Laparoscopic	57 (39-75)	16.0 (9.0-23.0)	3/0/5/8	14/2
Yin et al. (21)	2023	Retrospective	China	85	85	36 with laparoscopic and 49 with robotic	45 (32.5-57.5) and 49 (37-61)	17.0 (13.0-20.0) and 15.0 (13.0-20.0)	17/18/25/25	41/44
Roland et al. (22)	2008	Retrospective	USA	22	22	Laparoscopic	NR	NR	NR	NR
Sciuto et al. (23)	2014	Retrospective	Italy	9	10	Laparoscopic	36 (28-59)	18.0 (15.0-32.0)	0/0/4/6	NR

RFA, radiofrequency ablation; EA, ethanol ablation; NR, not reported; H/N/B/T, head/neck/body/tail; MIC-EN, mini-invasive enucleation.
*The tumor grade was unknown for the rest of the 20 patients.

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TABLE 2 Study endpoints of the included studies.

Author	Publication year	Study design	Country	Total patients, n	Number of lesions, n	Treatment approach	Severe AEs, n (%)	Mild AEs, n (%)	Technical success rate, %	Clinical success rate, %	Length of hospital stay days	Median follow-up period, month (range)	Symptom recurrence rates, n%
Debraine et al. (7)	2023	Retrospective	Belgium	11	11	RFA	0	4 (36.4)	100	90.9	NR	26 (9-53)	0
Oleinikov et al. (8)	2019	Retrospective	Israel	7	9	RFA	0	0	100	100	NR	9 (3-21)	0
Marx et al. (9)	2021	Retrospective	Switzerland	7	7	RFA	1 (14)	3 (43)	100	100	2.0 (1.0-3.0)	21 (3-38)	0
Sada et al. (10)	2023	Retrospective	US	8	8	EA	0	0	100	75	NR	43 (19.5, 81.5)	2 (25)
Yan et al. (11)	2022	Retrospective	China	9	10	EA	1 (7.1)	0	100	77.8	NR	33 (1-52)	5 (55.6)
Andreis et al. (12)	2023	Retrospective	Italy	10	10	RFA	0	2 (20)	100	100	NR	19.5 (12-59)	0
Jürgensen et al. (13)	2023	Retrospective	Germany	9	9	EA	0	1 (11.1)	100	100	4.0 (2.0-5.0)	17 (1-35)	1 (11.1)
Crinò et al. (14)	2023	Retrospective	Italy	89	89	RFA	0	16 (18.0)	100	95.5	3.5 (0.5-6.5)	23 (14-31)	15 (16.8)
Espan˜a-Go´mez et al. (15)	2009	Retrospective	Mexico	14	14	Laparoscopic	0	9 (64.3)	100	100	10.0 (2-21)	42 (1-90)	0
Belfiori et al. (16)	2018	Retrospective	Italy	15	15	MIC-EN	2 (13.3)	8 (53.4)	100	100	9 (5-54)	41 (1–134)	1 (6.7)
Cunha et al. (17)	2007	Retrospective	France	12	12	Laparoscopic	1 (8.3)	3 (25)	100	91.7	13 (7-20)	49 (20-78)	0
Hu et al. (18)	2011	Retrospective	China	43	43	Laparoscopic	5 (11.6)	8 (18.6)	100	95.3	9.0 (3.5-14.5)	6	0
Isla et al. (19)	2007	Retrospective	UK	21	21	Laparoscopic	1 (4.8)	2 (9.5)	100	100	5 (1–18)	NR	0
Nakamura et al. (20)	2015	Retrospective	Japan	15	16	Laparoscopic	2 (13.3)	0	100	100	12 (7–63)	43 (3–88)	0
Yin et al. (21)	2023	Retrospective	China	85	85	36 with laparoscopic	4 (11.1) and 3 (6.2)	32 (90.6) and	94.1	100	8.5 (6.0-11.3) and 6.0 (4.0-7.0)	65 (1-159)	2 (2.4)

(Continued)

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TABLE 2 Continued

Author	Publication year	Study design	Country	Total patients, n	Number of lesions, n	Treatment approach	Severe AEs, n (%)	Mild AEs, n (%)	Technical success rate, %	Clinical success rate, %	Length of hospital stay days	Median follow-up period, month (range)	Symptom recurrence rates, n%
						and 49 with robotic		45 (91.8)					
Roland et al. (22)	2008	Retrospective	USA	22	22	Laparoscopic	0	3 (13.6)	90.9	100	13.0 (8.5-17.5)	38.0 (33-43)	0
Sciuto et al. (23)	2014	Retrospective	Italy	9	10	Laparoscopic	1 (11.1)	2 (22.2)	100	100	7.0 (5-18)	45 (11-72)	0

RFA, radiofrequency ablation; EA, ethanol ablation; NR, not reported; AEs, adverse events.

3.3 Patient characteristics

In total, 391 lesions were identified from the 386 patients included in the 17 studies in the meta-analysis. Overall, 150 patients underwent EUS-guided ablation, whereby 26 (17.3%) underwent EUS-EA and 124 (82.7%) underwent EUS-RFA. The other 236 patients underwent minimally invasive surgery, among whom 52 (22.0%) underwent robotic surgery and 184 (78.0%) underwent laparoscopic surgery.

The pooled median age of the included patients was 53.05 years old (range: 22–97 years old); patients in the EUS group were older than those in the MIS group (64.06 vs. 44.98 years old, $p < 0.001$). Except for two studies (19, 22), the tumor size was reported in 15 studies. The pooled overall median tumor size was 14.74 mm (ranging from 3–52 mm), with no significant difference identified between the EUS and MIS groups (14.05 vs. 15.46 mm, $p = 0.303$). Except for one study (22), the other 16 studies (representing 369 lesions) reported the tumor location. In the EUS group, 67 lesions (43.8%) were located in the pancreatic head and neck, 57 (37.3%) in the pancreatic body, and 29 (18.9%) in the pancreatic tail. In the MIS group, 74 lesions (34.3%) were located in the pancreatic head and neck, 68 (31.4%) in the pancreatic body, and 74 (34.3%) in the pancreatic tail. The tumor distribution showed no significant difference between the two groups ($p = 0.063$). However, only 5 studies (7–9, 12, 14) in the EUS group (comprising 97 grade 1 lesions and 5 grade 2 lesions) and 2 studies (20, 21) in the MIS group (comprising 55 grade 1 lesions and 46 grade 2 lesions) reported the tumor grade; however, among those, the proportion of grade 1 lesions was significantly higher in the EUS group ($p < 0.001$) (Table 3).

3.4 Treatment-related adverse events

There was an unexpectedly high AE rate (98.8%) reported in the study by Yin et al. (21), and this was determined to be the origin of the heterogeneity in this analysis, so the study was removed from the further analysis of the AEs described below.

TABLE 3 Baseline characteristics and treatment outcomes of the included studies.

	EUS group	MIS group	*P-value*
Age, median years (range)	64.06 (28-97)	44.98 (22-84)	<0.001
Tumor size, median mm (range)	14.05 (6-21)	15.46 (7.5-52)	0.303
Tumor location, HN/BT, n	67/86	74/142	0.063
Tumor grade, Grade 1/2, n	97/5	55/46	<0.001
Adverse events, n (%)	28 (18.7)	47 (31.1)	0.012
Severe adverse events, n (%)	2 (1.3)	12 (7.9)	0.011
Technical success, n (%)	150 (100)	228 (96.6)	0.025
Clinical success, n (%)	141 (94)	3 (98.7)	0.021

H/N/B/T, head/neck/body/tail; MIS, minimally invasive surgery.

In the EUS group, 28 AEs (18.7%) occurred in 150 patients, including 2 severe AEs (1.3%). In the MIS group, 47 AEs (31.1%) occurred in 151 patients, including 12 severe AEs (7.9%). The AE rates and severe AE rates were significantly lower in the EUS group compared with the MIS group (p = 0.012 and 0.011) (Table 3).

3.5 Treatment outcomes

The technical success rate in the EUS group was 100%. However, 8 patients (3.4%) in the MIS group transferred to open surgery due to tumor location failure. The technical success rate in the MIS group was 96.6%. The technical success rate was significantly higher in the EUS group (p = 0.025). In terms of clinical success, 9 patients (6%) in the EUS group showed no symptom improvement after treatment, while 3 patients (1.3%) in the surgery group showed no symptom improvement after treatment. The difference between the two groups showed a statistical difference (p = 0.021) (Table 3).

Regarding hospital stay after treatment, only 3 studies (9, 13, 14) in the EUS group reported the length of hospital stay, while all the studies in the MIS group reported the length of hospital stay. The median length of hospital stay in the EUS group (2.68 days, 95%CI: 1.88–3.48, I^2 = 60.3%) was significantly shorter than that in the MIS group (7.40 days, 95%CI: 6.22–8.58, I^2 = 42.2%), with a *p-value* < 0.001 (Figure 2).

The pooled median follow-up time in the EUS group was 18.91 months (ranging from 1–81.5 months), while the pooled median follow-up time in the MIS group was 24.62 months (ranging from 1–159 months). The median follow-up time was similar in the two groups with no significant difference (p = 0.068) (Supplementary Figure 1). Also, 23 cases (15.3%) in the EUS group experienced symptom recurrence, but only 3 cases (1.3%) experienced recurrence in the MIS group. The recurrence rate was thus significantly lower in the MIS group (p < 0.001).

3.6 Sensitivity analysis

Sensitivity analysis was next performed to evaluate the stability of the AE results. According to the results of the sensitivity analysis (Supplementary Figures 2A, B), except for the study by Yin et al. (21), no study needed to be removed to maintain the stability of the results for the AEs rates in the EUS group (Supplementary Figure 2A) or the MIS group (Supplementary Figure 2B). We thus believe the results for the AE rates were stable.

3.7 Publication bias

Egger's tests were conducted for the reported AE rates to identify any potential publication bias. A possibility of publication bias (p = 0.008, Figure 3A) was indeed identified as related to the AE rates in the EUS group. However, the AE rate showed no publication bias in the MIS group (p = 0.082, Figure 3B).

4 Discussion

In this meta-analysis, the treatment outcomes of EUS-guided ablation and MIS were presented and compared. We confirmed that EUS-guided ablation was associated with fewer incidences of AEs, a shorter length of hospital stay, and a higher technical success rate. Although recurrence rate was significantly higher in the EUS group, the patients were much older in that group and therefore poorer surgical candidates, and so we consider EUS-guided ablation to be a

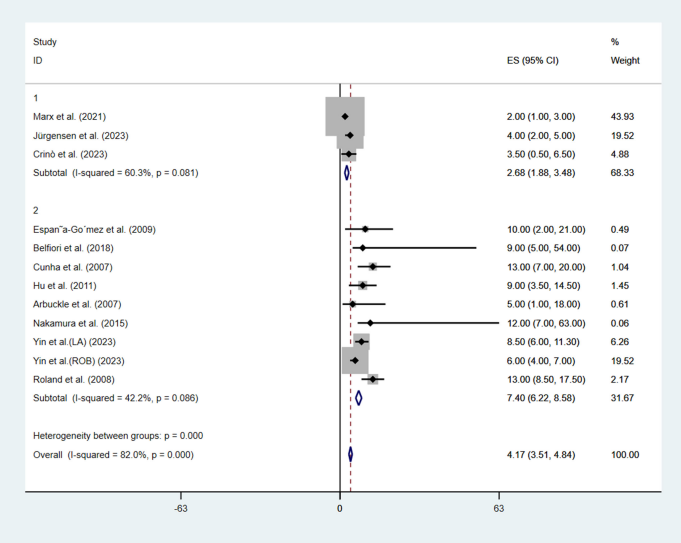

FIGURE 2
Pooled analysis of the length of hospital stay grouped by treatment method.

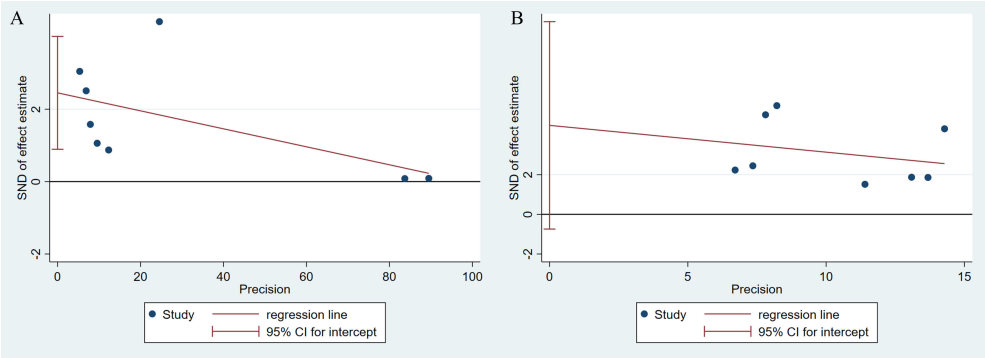

FIGURE 3
Egger's tests were conducted for assessing the rates of adverse events to identify potential publication bias. **(A)** EUS group, **(B)** Minimally invasive group (*p = 0.082*, **(B)**.

suitable alternative treatment approach for these and other poor surgical candidates.

Among all the panNETs, insulinomas are likely the best candidates for EUS-guided ablation because of their tiny size, minimal propensity for malignancy, and extremely quick symptom alleviation, which together make it simpler to track the effectiveness of treatment. However, EUS-guided ablation should not be performed in all insulinoma cases. Sporadic solitary insulinomas with a diameter of less than or equal to 20 mm, a minimum distance of 1 mm from the main pancreatic duct, and a Ki-67 value of less than 5% on EUS-guided cytology or from a biopsy sample may be the best candidates for this procedure, which was the condition considered in this meta-analysis. Moreover, the tumor sizes and tumor grades were found to be comparable between the EUS and MIS groups in this meta-analysis. The similar baseline characteristics of the included lesions indicate the results of this study are reliable.

The MIS treatment of insulinoma has gained widespread acceptance in the past decades. According to a meta-analysis, treating insulinomas through the laparoscopic approach is linked to a shorter hospital stay and comparable rates of postoperative AEs compared to open surgery (24, 25). However, for tumors located in the pancreatic head, pancreaticoduodenectomy is difficult to perform under MIS (26). Furthermore, to ensure full excision of the tumor, it is crucial to accurately identify the pancreatic resection line. As a result, guidance techniques, such as intraoperative ultrasound, should be used during MIS procedures (27). Considering this, additional expenses and operating time are required to provide proper surgical guidance. However, these limitations can be overcome by the use of EUS-guided ablation, whereby the tumor can be treated under real-time EUS guidance. Another meta-analysis concluded that the pooled sensitivity, specificity, and area under the ROC were 81%, 90%, and 0.92 for this approach (28). The results of that study indicated that EUS was an accurate approach for the preoperative localization of insulinomas (28). In our study, no difference in tumor distribution was observed. However, the *p-value* was very close to 0.05 (0.063), and the reported tumor locations may have been influenced by the subjective judgments of the radiologists and surgeons. We can conclude that for insulinomas located in the pancreatic head or neck, EUS-guided ablation is usually the preferred treatment method.

In 1999, Goldberg et al. (29) reported the first experimental EUS-RFA procedure in a pig model. They forecast the possible future application of EUS-RFA: "The development of endosonographically placed therapeutic devices may provide a unique alternative for the management of premalignant pancreatic lesions and potentially may offer palliative therapy for surgically unresectable malignant pancreatic tumors." In 2006, Jürgensen et al. first reported the use of EUS-EA in treating an insulinoma, in which a 78-year-old patient achieved a durable, complete remission of their tumor (30). Several case reports and case series reports have confirmed the potential advantages of EUS-guided ablation in treating insulinomas. Recently, a meta-analysis with 19 studies and 183 patients (comprising 101 functional panNETs and 95 non-functional panNETs) summarized that the pooled overall AE rates for clinical efficacy were 17.8% and 95.1% for functional panNETs and 24.6% and 93.4% for non-functional panNETs. These results were very similar to those in our study. Another meta-analysis explored the safety and efficacy of EUS-guided ablation for solid pancreatic tumors. The AE rates were 32.2% for RFA and 21.2% (95% CI: 6.8–49.9%) for EA (31), and severe complications rarely occurred. However, the studies included in those meta-analyses were all single-arm studies. Comparative studies are required to verify the true value of EUS-guided ablation and MIS in the treatment of insulinomas. To the best of our knowledge, our study is the first comparative meta-analysis to compare the clinical outcomes of EUS and MIS approaches for treating insulinomas.

The high recurrence rate associated with EUS-guided ablation may be a concern for its wider clinical application. The reason for this high recurrence rate may be due to the fact that endoscopic ablation is more likely to have residual tumor cells than surgical

resection, but no study has yet confirmed this hypothesis. Regarding the recurrence or advancement of insulinomas treated with EUS-guided ablation, the long-term results are still unclear. Our study first reported the long-term outcomes of EUS-guided ablation for the treatment of insulinoma. The recurrence rate was 15.6% after a median 18-month follow-up. Considering the recurrence rate was acceptable and the procedure can be repeated after recurrence, although the recurrence rate was higher, we still believe that EUS-guided ablation is a valuable approach. However, significant heterogeneity was identified among the different studies in our meta-analysis. Prospective studies with a larger sample size are warranted to verify the true long-term outcomes of EUS-guided ablation.

Undoubtedly, there were still some limitations of our study to note. First, the heterogeneity between studies caused by the methodological and clinical diversities was high. All the included studies were retrospective, so significant selection bias may exist. Second, all the studies compared the two treatment methods directly. The superiority of the two treatment methods should be verified in a further prospective randomized controlled study. Third, the different outcomes of EUS-RFA and EUS-EA were not analyzed due to the small sample sizes. However, the treatment mechanisms are different between EUS-RFA and EUS-EA, and the outcomes may be different. Fourth, the expression of data among different studies was different. Some studies expressed data as the median, and some expressed data as the mean. We estimated some baseline data to make the expressions consistent, which may have caused bias. Fifth, publication bias still existed in our study. The overwhelming predilection of sponsors, periodicals, and researchers to look for optimal outcomes was the main source of this publication bias. Furthermore, another element that contributed to publication bias was the considerable between-study heterogeneity. Sixth, although not statistically significant, the follow-up time was shorter in the EUS group in our study, which could have influenced the relapse rate.

5 Conclusion

We conducted a meta-analysis and systematic review to compare the treatment outcomes of EUS-guided ablation and MIS for the treatment of insulinoma. We identified that the age of the patients in EUS-guided ablation was associated with lower AE rates and lower severe AE rates, and a shorter length of hospital stay, but a higher recurrence rate after treatment. EUS-guided ablation may be an alternative, and even first-line, treatment for poor surgery candidates. Further prospective studies comparing the two treatment methods are warranted to establish the true role of EUS-guided ablation in the treatment of insulinomas.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

DX: Conceptualization, Formal analysis, Writing – original draft. LZ: Data curation, Formal analysis, Software, Writing – review & editing. SX: Formal analysis, Validation, Writing – original draft. XY: Data curation, Resources, Writing – review & editing. QJ: Formal analysis, Writing – original draft. AW: Writing – review & editing. YJ: Conceptualization, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1367068/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Pooled analysis of the median follow-up time grouped by treatment method.

SUPPLEMENTARY FIGURE 2
Sensitivity analysis results of adverse events. **(A)** EUS-guided ablation, **(B)** Minimally invasive surgery.

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EDITED BY
Bojana Popovic,
University of Belgrade, Serbia

REVIEWED BY
Karin Zibar Tomsic,
University Hospital Centre Zagreb, Croatia
Sam Zuber,
Surgical Associates of La Jolla, United States

*CORRESPONDENCE
Madson Q. Almeida
madson.a@hc.fm.usp.br

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Intraoperative radiofrequency ablation for unresectable abdominal paraganglioma: a case report

Isabelle P. A. Magalhaes[1], Bibiana D. Boger[1], Nathalia L. Gomes[2,3], Guilherme L. P. Martins[4], Leomarques A. Bomfim Jr.[5], Gustavo F. C. Fagundes[1], Roberta S. Rocha[2], Fernando M. A. Coelho[5], Jose L. Chambo[6], Ana Claudia Latronico[1], Maria Candida B. V. Fragoso[7,8], Ana O. Hoff[8], Berenice B. Mendonca[7], Marcos R. Menezes[4] and Madson Q. Almeida[1,8*]

[1]Adrenal Unit, Laboratory of Molecular and Cellular Endocrinology, LIM/25, Division of Endocrinology and Metabolism, Clinics Hospital, University of Sao Paulo Medical School, Sao Paulo, Brazil, [2]Division of Endocrinology, Santa Casa de Belo Horizonte, Belo Horizonte, Brazil, [3]Department of Internal Medicine, Federal University of Minas Gerais Medical School, Belo Horizonte, Brazil, [4]Interventional Radiology, Cancer Institute of São Paulo State (ICESP), University of Sao Paulo Medical School, Sao Paulo, Brazil, [5]Radiology Institute InRad, Clinics Hospital, University of Sao Paulo Medical School, Sao Paulo, Brazil, [6]Division of Urology, Clinics Hospital, University of Sao Paulo Medical School, Sao Paulo, Brazil, [7]Adrenal Unit, Laboratory of Hormones and Molecular Genetics LIM/42, Division of Endocrinology and Metabolism, Clinics Hospital, University of Sao Paulo Medical School, Sao Paulo, Brazil, [8]Division of Endocrine Oncology, Cancer Institute of São Paulo State (ICESP), University of Sao Paulo Medical School, Sao Paulo, Brazil

For pheochromocytoma and paraganglioma (PPGL), the efficacy of percutaneous ablative therapies in achieving control of metastatic tumors measuring <3 cm had been demonstrated in only few reports, and intraoperative radiofrequency ablation (RFA) of locally invasive primary PPGLs has not been reported. We presented the case of a 31-year-old man who had a 9-cm functioning unresectable PPGL. He was treated with 13 cycles of cytotoxic chemotherapy without objective tumor response, according to the Response Evaluation Criteria in Solid Tumors (RECIST). Subsequently, magnetic resonance imaging revealed a 9.0 × 8.6 × 6.0-cm retroperitoneal mass that extended to the inferior portion of the inferior vena cava, the inferior mesenteric artery, and the infrarenal aorta. Biochemical evaluation demonstrated high level of plasma normetanephrine (20.2 nmol/L, normal range <0.9 nmol/L). Genetic investigation showed the germline pathogenic variant c.1591delC (p. Ser198Alafs*22) in the *SDHB* gene. I^{131}-metaiodobenzylguanidine scintigraphy was negative and Ga^{68}-dotatate PET-CT scan showed high tumor uptake without distant metastases. On open laparotomy, tumor debulking was not possible. Therefore, intraoperative RFA was performed by a highly experienced team of interventional radiologists. At 12 months after the RFA, the tumor volume decreased from 208 to 45 mL (78%), plasma normetanephrine decreased from

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20.2 to 2.6 nmol/L (87%), and the doxazosin dose was reduced from 16 to 8 mg/day. To our best knowledge, this was the first report on intraoperative RFA that markedly reduced the size of a large primary unresectable PPGL, along with clinical and biochemical responses.

KEYWORDS

paraganglioma, radiofrequency, ablation, intraoperative, unresectable

Introduction

Metastatic pheochromocytomas and paragangliomas (PPGLs) are defined by the presence of distant metastases at sites where chromaffin cells are physiologically absent (1). The latest World Health Organization (WHO) classification considered all PPGLs are considered to have metastatic potential, and replaced the previous term "malignant or benign" to metastatic or not metastatic (2). Approximately 10% to 15% of pheochromocytomas and a higher proportion (35% to 40%) of paragangliomas develop metastatic lesions (1). Large tumors >5 cm, paragangliomas (extra-adrenal location), multifocal disease, high plasma methoxityramine levels (normal range <0.1 nmol/L), normetanephrine level >5x the upper limit of normal reference range, and germline *SDHB* pathogenic variants had been associated with a higher risk for metastatic disease (3–6). Furthermore, patients with locally advanced pheochromocytoma (i.e., with capsular, vascular, and adipose tissue invasion) and/or positive locoregional lymph nodes butwithout evidence of distant secondary lesions at the time of diagnosis have an increased risk of recurrence (7).

Surgical removal is the main strategy to treat PPGLs (8, 9). The first choice treatment for patients with slow to moderate progression of metastatic and/or unresectable disease is radionuclide therapy with conventional or high-specific-activity ^{131}I-metaiodobenzylguanidine (MIBG) or ^{177}Lu-DOTATATE (10). Even in patients with metastatic disease, primary tumor resection appears to be associated with better blood pressure control and improved overall survival (11, 12). Radiation therapy and interventional radiology techniques can help control the symptoms of local metastases (especially bone lesions) to control local symptoms, and can also be effective for unresectable PPGL (13). However, there had been no studies that specifically addressed neoadjuvant MIBG or ^{177}Lu-DOTATATE therapy for unresectable PPGL.

In a retrospective series, computed tomography (CT)-guided percutaneous ablative therapy by radiofrequency or cryoablation of 32 metastatic PPGLs, majority of metastatic lesions were in the bones and liver measuring up to 3 cm, were shown to effectively promote local control and palliate symptoms (14). However, intraoperative radiofrequency ablation (RFA) of large and unresectable primary PPGLs has not been previously demonstrated. Here, we reported the clinical, biochemical, and radiological outcome of an unresectable abdominal paraganglioma treated with intraoperative RFA.

Case report

A 31-year-old male patient presented with symptoms of catecholamine excess, (such as sweating, weight loss, and poor control of hypertension), abdominal pain, and a retroperitoneal tumor. He was previously diagnosis of arterial hypertension at 18 years of age, but with no regular medical follow-up. Urinary normetanephrine level was 4260 ug/24h (normal range <732 µg/24h). Abdominal CT revealed a 10.5 x 7.2 x 9.5 cm retroperitoneal mass, which involved the inferior vena cava, inferior mesenteric artery, and infrarenal aorta. The patient underwent open laparotomy at another medical center, where an unresectable mass was revealed. Anatomopathological analysis confirmed the diagnosis of paraganglioma. Immunostaining was positive for chromogranin A, synaptophysin, GATA3 and Ki67 (5% to 10%). He was treated with 13 cycles of cytotoxic chemotherapy (cyclophosphamide, vincristine and dacarbazine) without objective tumor response, according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria.

The patient was then referred to our Institution for possible additional therapies. His blood pressure was well controlled with doxazosin 16 mg/day, propranolol 40 mg/day and losartan 100 mg/day. Magnetic resonance imaging (MRI) revealed an extensive retroperitoneal mass, which measured 9.0 x 8.6 x 6.0 cm (208 mL) and involved the inferior portion of the inferior vena cava, inferior mesenteric artery, and infrarenal aorta (Figure 1A). Biochemical evaluation demonstrated very high levels of plasma normetanephrine (20.2 nmol/L, normal range <0.9 nmol/L) and normal metanephrine level (0.2 nmol/L, <0.5 nmol/L). The cathecolamine metabolites were measured using liquid chromatography–tandem mass spectrometry.

Genetic investigation showed the germline pathogenic variant c.1591delC (p.Ser198Alafs*22) in the *SDHB* gene. I^{131}-MIBG scintigraphy did not show any tumor uptake. The Ga^{68}-DOTATE PET-MRI showed a high uptake, with a maximum standardized uptake value (SUV max) of 32.5 in the abdominal mass, with local invasion but without distant metastatic disease (Figure 2A). Because of the lack of alternative therapies, we proposed debulking by open laparotomy. Notably, the tumor did not have a clear cleavage plane with the large vessels and had a large caliber intratumoral vasculature. Therefore, intraoperative RFA was performed on the remaining lesion to promote tumor debulking. Intraoperative

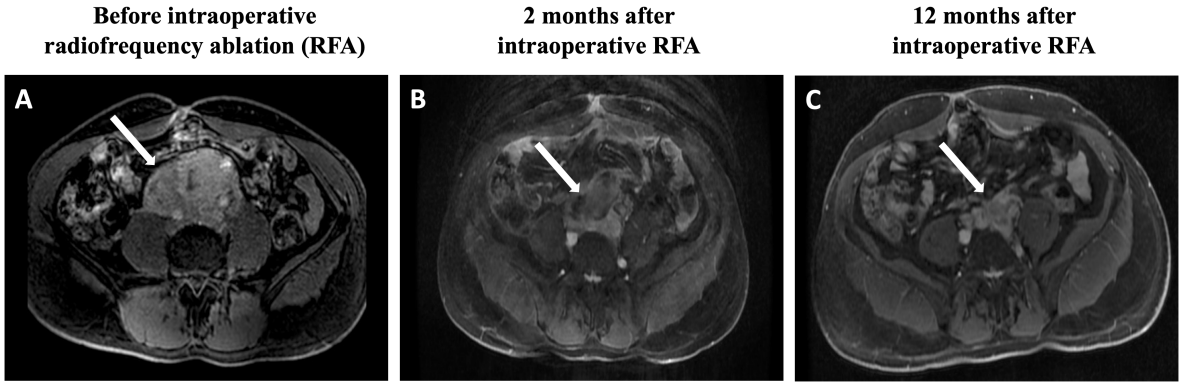

FIGURE 1
Magnetic resonance imaging findings: **(A)** Postgadolinium fat-saturation axial T1 weighted images reveal an extensive retroperitoneal mass, which measures 9.0 × 8.6 × 6.0 cm and involves the inferior portion of the vein inferior vena cava, inferior mesenteric artery, and infrarenal aorta. **(B)** The tumor size is reduced to 6.9 × 6.8 × 5.4 cm. **(C)** After 12 months of the RFA procedure, the tumor further shrinks to 7.2 × 4.8 × 4.1 cm. RFA, radiofrequency ablation.

biopsy confirmed the diagnosis of paraganglioma with immunostaining positive for chromogranin and synaptophysin.

Intraoperative RFA was performed by a very experienced team, who used a Cool-tip™ RF E series ablation cluster electrode (Medtronic), which was 15.0-cm long and had a 2.5-cm active tip, under guidance by intraoperative ultrasound (GE healthcare LOGIQ™). Eight rounds were performed using an automatic protocol with maximum output power of 200 W lasting 6-12 min each, according to the behavior/impedance which ranged from 80 to 110 ohms during the procedure, in order to contemplate the middle and anterior portions of the lesion, reaching approximately 80% of the tumor volume. The left posterior portions of the lesion were only partially ablated because of their proximity to the left ureter. The oral anti-hypertensive medications (doxazosin, propranolol and losartan) were discontinued on the day of the procedure. During the procedure, invasive hemodynamic monitoring was performed by invasive arterial and central venous pressure monitoring. The patient's blood pressure was controlled intra-operatively with continuous intravenous sodium nitroprusside. There were no reported immediate complications in the intra- and postoperative period. After the procedure, no active bleeding was observed, and there was a partial reduction in the tumor volume associated with areas of coagulative necrosis/charring in the periphery.

After 2 months of the RFA procedure, the dose of doxazosin was decreased from 16 to 8 mg (50% reduction), the plasma normetanephrine level dropped to 4.6 nmol/L (79% reduction), and the tumor size decreased to 6.9 x 6.8 x 5.4 cm (88.8 mL, 57.3% reduction in volume) on MRI (Figure 1B). After 12 months, the tumor further shrunk to 7.2 x 4.8 x 4.1 cm (45 mL, 78.4% reduction in volume), and plasma normetanephrine decreased to 2.9 nmol/L

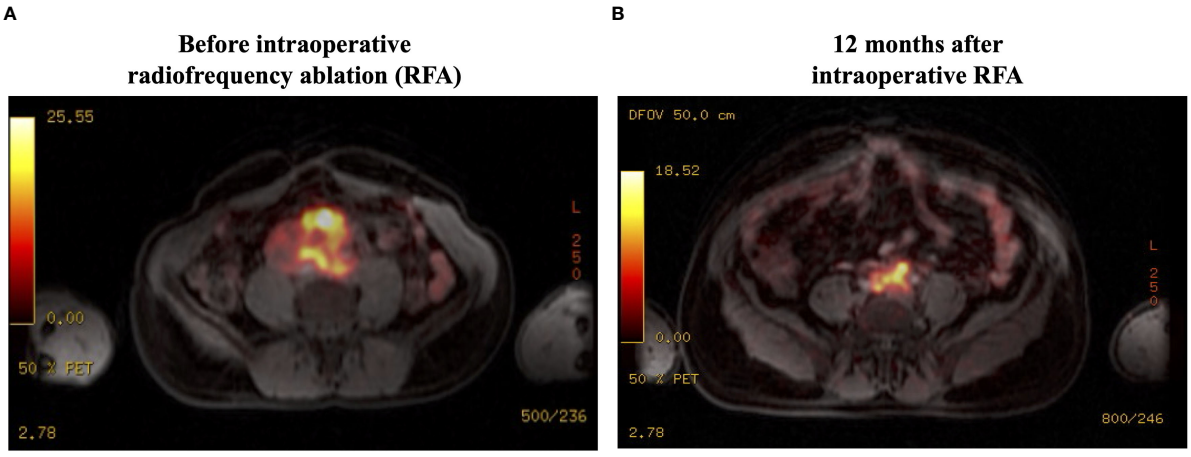

FIGURE 2
Ga^{68}-DOTATATE PET-MRI findings: **(A)** There is high uptake (SUVmax, 32.5) in the abdominal mass, with local invasion but without distant metastatic disease. **(B)** There is a significant reduction in the tumor uptake (SUVmax, 18.5) at 12 months after RFA. SUVmax, maximum standardized uptake value; RFA, radiofrequency ablation.

(86% reduction) (Figure 1C). Ga^{68}-dotatate PET-MRI showed a significant reduction in the SUVmax from 32.5 to 18.5 after 12 months of the RFA (Figure 2B).

Discussion

Compared with other solid tumors, metastatic and/or unresectable PPGLs are more indolent and have an estimated 5-year survival rate of 34% to 74% (15). Approximately 35% of the metastatic PPGLs have synchronous metastases upon initial diagnosis (10). The most frequent sites of metastases are the bone and lymph nodes (2). Lung and liver metastases are associated with poor outcome (15). The approach to systemic disease is mainly palliative and divided by two main scenarios: rapid progression vs. slow or moderate progression. Rapid progression is defined by a high tumor burden for <6 months, especially if there are secondary lesions in the liver and lung. The first choice treatment for cases of slow to moderate progression is radionuclide therapy (13). In fact, treatment with ^{131}I-MIBG (dose ranging from 492 to 1,160 mCi) promote a 22% partial objective response according to the RECIST 1.1 (16). However, 35% of patients showed disease progression after 1 year. A prospective trial showed that high-specific-activity ^{131}I-MIBG (500 mCi twice at 3 to 6 month intervals) was associated with a 30% partial response rate and 68% stable disease rate after a second dose, based on RECIST (17). Furthermore, a 50% reduction in all antihypertensive drugs, lasting at least 6 months, occured in 25% of the patients.

In the case reported here, the paraganglioma did not show any uptake on I^{131}- MIBG scintigraphy. The patient had no objective tumor response after 13 cycles of cytotoxic chemotherapy (cyclophosphamide, vincristine and dacarbazine). Indeed, stable disease had been the most frequent outcome of metastatic PPGLs after the available systemic therapies (13). Because the Ga^{68}-DOTATE PET-MRI showed a positive uptake, ^{177}Lu-DOTATATE would have been a treatment option, but this radionuclide therapy is not available in the public health system in Brazil. The largest study to date reported an overall response rate of 23% after ^{177}Lu-DOTATATE in 30 patients with metastatic PPGLs (18). Moreover, a metanalysis showed that treatment with ^{90}Yor ^{177}Lu-based peptide receptor radionuclide therapy achieved an objective response rate of 25% and a disease control rate of 84%. Clinical and biochemical responses were seen in 61% and 64% of the patients, respectively (19).

For this present case, a debulking open laparotomy was proposed by a highly experienced surgeon because of the lack of alternative therapies, but it was not anatomically feasible. The main indication for RFA was the presence of large vessel invasion (inferior vena cava, inferior mesenteric artery, and infrarenal aorta). Therefore, intraoperative RFA was performed and led to significant objective tumor and biochemical responses. Percutaneous RFA has been used for primary pheochromocytomas (non-invasive and non-metastatic) in patients who are poor candidates for surgery (20–22). Although percutaneous ablative therapies can be used for the local management of small metastatic lesions, to the best of our knowledge, intraoperative RFA of an unresectable PPGL has not been previously reported.

Both intraoperative and percutaneous RFA have been increasingly applied in the management of unresectable pancreatic cancer, with a high clinical success rate (23). In addition, intraoperative or percutaneous RFA was shown to be safe, well-tolerated, and effective treatment in achieving destruction in patients with unresectable primary and metastatic hepatic malignancies (22). Curley et al. (22) used RFA to treat 169 tumors (median diameter 3.4 cm, range 0.5 to 12 cm) in 123 patients with primary liver cancer (39%) or metastatic liver lesions (61%). After a median follow-up of 15 months, tumor has recurred in only 3 of 169 treated lesions (1.8%). The efficacy and safety of CT-guided ablative therapy for metastatic PPGLs has been demonstrated in small series (24, 25). McBride et al. (25) evaluated the efficacy of percutaneous ablation in 10 patients with metastatic PPGLs. Most of the lesions were located in the liver and bones with mean tumor size of 2.1 cm (range, 0.5 to 4.6 cm). Successful ablation without evidence of recurrence was achieved in 56% (15 of 27) of the primarily treated lesions in patients who had available follow-up imaging (25). In 2019, Kohlenberg et al. (14) expanded this series from Mayo Clinic and studied 123 lesions from 32 patients with metastatic PPGL were treated with percutaneous CT-guided RFA or cryoablation. Radiological local control was achieved in 86% of the lesions. Clinical improvement (pain or symptoms of catecholamine excess) was achieved in 12 of 13 (92%) procedures, with need for intravenous blood pressure medications in 14% of the procedures and procedure-related death in only 1 patient (14).

RFA using microwave ablation for larger tumors may represent a very effective treatment for large tumors. Microwave ablation offers advantages such as faster treatment times, larger ablation zones, no heat sink effect in tumors close to large vessels and potentially improves success rates. Further research and clinical studies are warranted to explore this approach.

In conclusion, we reported the first intraoperative RFA for a large primary unresectable PPGL, with marked clinical, biochemical, and tumor responses. Blood pressure was safely controlled with intravenous medication during the procedure. The patient did not have any serious complications after RFA. Therefore, we propose this novel, effective, and safe approach for debulking a large and unresectable primary PPGL.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Written informed consent was obtained from the patient to perform genetic analysis and for the publication of any potentially identifiable images or data included in this article.

Author contributions

IM: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. BB: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. NG: Data curation, Validation, Writing – review & editing. GM:

Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology, Software. LB: Investigation, Methodology, Software, Writing – review & editing. GF: Formal analysis, Investigation, Supervision, Writing – review & editing. RR: Formal analysis, Investigation, Supervision, Writing – review & editing. FC: Supervision, Writing – review & editing, Investigation, Software. JC: Investigation, Supervision, Writing – review & editing. AL: Conceptualization, Supervision, Validation, Writing – review & editing. MF: Supervision, Validation, Visualization, Writing – review & editing. AH: Conceptualization, Supervision, Validation, Writing – review & editing. BM: Conceptualization, Formal analysis, Supervision, Writing – review & editing. MM: Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – review & editing. MA: Conceptualization, Supervision, Writing – review & editing.

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Conflict of interest

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

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EDITED BY
Claire Perks,
University of Bristol, United Kingdom

REVIEWED BY
Pawel Licznerski,
Yale University, United States
Smrithi Padmakumar,
Spark Therapeutics Inc, United States

*CORRESPONDENCE
Michael Sigal
michael.sigal@charite.de
Bertram Wiedenmann
bertram.wiedenmann@charite.de

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Elevated sortilin expression discriminates functional from non-functional neuroendocrine tumors and enables therapeutic targeting

Felix Bolduan[1,2], Alexandra Wetzel[1], Yvonne Giesecke[1], Ines Eichhorn[1], Natalia Alenina[3,4], Michael Bader[3,4,5,6], Thomas E. Willnow[3,7], Bertram Wiedenmann[1]* and Michael Sigal[1,8]*

[1]Department of Hepatology & Gastroenterology, Charité Universitätsmedizin Berlin, Berlin, Germany, [2]Berlin Institute of Health at Charité – Universitätsmedizin Berlin, BIH Biomedical Innovation Academy, BIH Charité Junior Digital Clinician Scientist Program, Berlin, Germany, [3]Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany, [4]German Center for Cardiovascular Research (DZHK), Berlin, Germany, [5]Charité - Universitätsmedizin Berlin, Berlin, Germany, [6]University of Lübeck, Institute for Biology, Lübeck, Germany, [7]Department of Biomedicine, Aarhus University, Aarhus, Denmark, [8]Berlin Institute for Medical Systems Biology (BIMSB), Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany

A subset of neuroendocrine tumors (NETs) can cause an excessive secretion of hormones, neuropeptides, and biogenic amines into the bloodstream. These so-called functional NETs evoke a hormone-related disease and lead to several different syndromes, depending on the factors released. One of the most common functional syndromes, carcinoid syndrome, is characterized mainly by over-secretion of serotonin. However, what distinguishes functional from non-functional tumors on a molecular level remains unknown. Here, we demonstrate that the expression of sortilin, a widely expressed transmembrane receptor involved in intracellular protein sorting, is significantly increased in functional compared to non-functional NETs and thus can be used as a biomarker for functional NETs. Furthermore, using a cell line model of functional NETs, as well as organoids, we demonstrate that inhibition of sortilin reduces cellular serotonin concentrations and may therefore serve as a novel therapeutic target to treat patients with carcinoid syndrome.

KEYWORDS

neuroendocrine tumors, functional syndrome, carcinoid syndrome, serotonin, sortilin, organoids, enteroendocrine cells

Introduction

Neuroendocrine tumors (NETs) are rare malignant neoplasms that can occur as primary tumors in every organ but are most frequently found in the gastroenteropancreatic (GEP) system. NETs consist of cells displaying neuronal and endocrine characteristics (1, 2). Since 1975, the age-adjusted incidence rate of GEP-NETs in the US has increased by 6.4 times to 5.45 per 100,000 persons (3). In approximately one-third of cases, NETs secrete excessive amounts of peptidic hormones and biogenic amines into the circulation, resulting in a hormone-mediated disease. These tumors are called "functional". One of the most common hormone-mediated disease is carcinoid syndrome, caused primarily by over-secretion of serotonin, which occurs in 32% of all small intestinal NETs (4). Metastatic grade small intestinal NET patients suffering from this syndrome have a significantly shorter median overall survival (4.7 years, Confidence interval 4.0-5.4) than those without functional syndrome (7.1 years, CI 5.2-8.1) (4, 5). It is widely accepted that in order to cause carcinoid syndrome, cells need to evade the first-pass effect of the liver (e.g., due to liver metastases, widespread retroperitoneal metastases, ovarian metastases, or bronchial primaries). However, morphologically it is not possible to discriminate functional from non-functional tumors and comparative molecular analyses have so far failed to reveal obvious cellular markers of functional tumors. Identification of specific features of functional cells may not only provide insights into the tumor biology of NETs but also reveal novel and highly needed therapeutic targets.

Sortilin, also known as neurotensin receptor 3, is a member of the VPS10P domain receptor family - a group of transmembrane receptors involved in uptake as well as intracellular sorting of a broad range of protein ligands (6). Recent studies focusing on the role of sortilin in cancer uncovered that this receptor is expressed in many cancer cells, including breast and lung cancer. Its expression has been associated with a variety of effects, ranging from pro-tumorigenic to tumor-suppressive (7–9). Expression of sortilin was also detected in NETs and linked to cell migration and adhesion (10). Interestingly, only a fraction of tested NET samples was positive for sortilin expression, raising the question of what distinguishes sortilin-positive and sortilin-negative tumors. As sortilin plays an important role in regulating secretion of neurotrophins in neurons (11, 12), we hypothesized that this receptor may be essential for the ability of NETs to produce and secrete hormones and thereby serves as a key factor in the development of functional syndrome.

In this study we aim to demonstrate that elevated sortilin expression is a novel feature of hormonally active NETs. By comparative sortilin-immunostaining we could indeed confirm this hypothesis. Furthermore, we demonstrated that sortilin inhibition causes reduced levels of serotonin in cell culture systems. Thereby, sortilin serves as a novel potential target in treatment of the functional syndrome of NETs.

Methods

Neuroendocrine tumor tissue

Paraffin-embedded tissue of neuroendocrine tumor samples was used for this study. The collection of tissue samples for this study was approved by the Ethics Committee of the Charité - Universitätsmedizin Berlin (No EA1/229/17). 1 mm punches with a thickness of 2 µm were obtained and plated on tissue microarrays (TMA). For each sample, 3 replicates were taken. Tissue was rehydrated, antigen retrieval was performed using 10 mM citrate buffer and incubated for 20 min in Avidin/Biotin Blocking Solution (Dako, X0590). Slides were washed in phosphate-buffered saline (PBS, Gibco) and incubated for 10 min in 0.1% Triton X-100 in PBS. After further washing steps, the tissue was incubated overnight at 4°C with anti-sortilin antibody (1:100, af3154, R&D systems). After repeated washing steps, tissue was incubated for 30 min at room temperature with a secondary biotin-linked anti-goat antibody (1:300, Dako, E0466), followed by incubation with ABC complex solution (Vector, PK-6100) for 30 min. After an additional washing step with PBS, a DAB buffer solution (Dako, 3468) was applied according to the manufacturer's instruction until a color change occurred (approx. 10 min) before stopping the reaction with distilled H2O. Staining with hematoxylin was performed and tissue embedded in glycerin-gelatine.

In total, 49 tumor samples [21 neuroendocrine primary tumors (7 pancreatic NETs and 14 small intestinal NETs; of these 14 functional and 7 non-functional) and 28 liver metastases of neuroendocrine tumors (10 pancreatic, 1 lung, 15 small intestinal, and 2 NETs of unknown origin; of these, 16 functional and 12 non-functional)] were included in this study. To exclude the possibility of high hormonal secretion without symptoms in small intestinal NETs due to the first-pass effect of the liver, only primary tumors that caused hepatic metastases were included. Tissue was examined under brightfield microscopy and immunoreactivity scores were obtained by multiplication of the staining intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and the percentage of positively stained cells (0, no positive cells; 1, 0%-10% positive; 2, 11%-50% positive; 3, 51%-100% positive). Each sample consisted of three replicates and the final score for each sample was obtained by averaging the immune reactivity scores of all three. Negative controls without primary antibody were established for every sample.

Cell culture

BON cells, a kind gift from Courtney Townsend (University of Texas, Galveston, TX), were cultured in RPMI 1640 medium containing L-glutamine (Gibco) supplemented with 10% FBS at 37°C with 5% CO2 in O2. During the exponential growth phase, cells were treated with the sortilin inhibitor AF38469 (10 µM, MedChemExpress) for 24 h. In order to exclude toxic effects, cell viability measurements were performed. After 24 h incubation with 10 µM AF38469 or vehicle, cells were harvested and counted using Countess II™ (Invitrogen).

They were further treated with trypan blue (Invitrogen) and alamarBlue™ (Thermo Fisher Scientific) to determine the percentage of live cells and cell proliferation, respectively.

Western blot assay

NET tissue or BON cells were washed with PBS (Gibco), lysed with RIPA buffer (Thermo Fischer Scientific) containing a protease inhibitor cocktail (cOmplete™ Mini, Roche) and sonication (10s, 60% intensity). The proteins were separated by SDS-PAGE (10-12% Tris-Glycin, WedgeWell™, Invitrogen) and transferred onto PVDF membranes (Bio-Rad). After blocking with 5% nonfat dry milk, the membranes were incubated with primary antibodies against sortilin (1:250, AK BD#612101, Becton Dickenson) and α-tubulin (1:1000, #T9026, Sigma) overnight at 4°C, followed by incubation with secondary anti-mouse IgG antibody (1:10000, #AB_2340061, Jackson Immuno Research) for 1 h at room temperature. Detection was performed with SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific) using the Molecular Imager® VersaDoc™ and quantified with Image Lab™ software (Bio-Rad).

Primary mouse small intestinal organoid culture

Experiments and animal maintenance were performed in accordance with local (LaGeSo, Berlin, T-CH0032/20), national (German Animal Welfare Act), and international guidelines (EU Directive 2010/63/EU). Male 6 to 12-week-old C57BL/6 mice obtained from Charles River Laboratory were used for this study. For the generation of murine small intestinal organoids, animals were sacrificed by cervical dislocation, the ileum was dissected, washed with ice-cold PBS (Gibco), and opened longitudinally. The villi were gently removed using a glass coverslip. 5 mm-long pieces were washed 10 times in ice-cold PBS, followed by incubation for 5 min in 10 mM EDTA (Invitrogen)/PBS at room temperature and 30 min in 2 mM EDTA/PBS supplemented with 2.5 µM DTT (Sigma) at 4°C on a rotating shaker. After removing the supernatant, the pieces were shaken vigorously with 5 ml HBSS containing magnesium and calcium chloride (Gibco) and 10 ml PBS. The supernatant was filtered through a 70 µm filter (Greiner Bio-One) and centrifuged at 200 x g for 3 min at 4°C. The pellet was resuspended with 3 ml 0.1% BSA/PBS and the number of crypts determined. After a repeated centrifugation step, 2,000 crypts per 10 µl Cultrex Basement Membrane Extract Type 2 (R&D systems) were seeded in 48-well plates. The organoids were cultured in Advanced Dulbecco's Modified Eagle's Medium/F12 (Gibco) consisting of 10 mM HEPES (Gibco), 2 mM GlutaMAX (Gibco), and 10% penicillin/streptomycin (Gibco), supplemented with 1.25 mM N-acetylcysteine (Sigma), 25% R-spondin 1 conditioned medium, 1× B-27 (Gibco), 1× N2 (Gibco), 50 ng/ml mEGF (Invitrogen), and 100 ng/ml mNoggin (PeproTech). The medium was replaced every 2-3 d and the organoids were passaged mechanically after 5-7 d using a syringe and needle.

EEC differentiation

Three days after passaging, ileal organoids were treated with the above-described medium but without mEGF and supplemented with a Notch inhibitor (10 µM DAPT, Sigma) and a MEK1 and 2 inhibitor (1 µM PD0325901, Sigma) for four days. To confirm the enrichment of EECs, expression of the marker synaptophysin was determined using immunofluorescence staining. Organoids were harvested, washed with PBS, and fixed with 3.7% paraformaldehyde overnight at 4°C, followed by incubation in 0.1% BSA/PBS for at least 30 minutes. After embedding in 2% agarose, organoids were dehydrated, embedded in paraffin, and cut into 4 µm sections. Sections were deparaffinized and rehydrated, followed by antigen retrieval in 10 mM citrate buffer (pH 6). To avoid unspecific antibody binding, sections were incubated with blocking buffer (0.1% Tween/PBS supplemented with 5% FBS) for 2 h at room temperature, followed by incubation with the primary antibody against synaptophysin (1:100, ab178412, clone EPR1097-2, Abcam) overnight at 4°C. Sections were washed three times with 0.1% Tween/PBS, incubated with the secondary antibody AlexaFluor 647 donkey anti-rabbit IgG (1:400, Jackson Immunoresearch, 711-606-152) and DAPI for 2 h at room temperature, mounted by using an aqueous mounting medium and covered with a glass cover slip.

Immunofluorescence staining of serotonin

For immunofluorescence staining of serotonin after sortilin inhibition, 2,000 BON cells were trypsinized, seeded onto glass slides, and fixed with methanol/acetone (1:1) for 2 min at room temperature. After washing with PBS and blocking with 5% goat serum in PBS (Biochrom) for 30 min at room temperature, the cells were incubated with the primary antibody against serotonin (1:400, #M0758, clone 5HT-H209, Dako) for 1 h at room temperature. The cells were washed three times for 2 min with PBS and incubated with Cy3-Goat anti-mouse IgG (1:1000, Jackson Immuno Research, 111-225-144) for 30 min at room temperature. After repeated washing with PBS, the cells were incubated with ethanol for 2 min and mounted in Immu-Mount™ (#9990402, Epredia).

All immunofluorescence images were acquired with the confocal laser scanning microscope TCS SP8 (Leica) or Observer 7 microscope (Zeiss). Images were collected and analyzed with Leica Application Suite X 3.5.6.21594 (LAS X, Leica) and ZEN 3.4 (Zeiss). Five images were collected per sample.

Serotonin quantification

For quantification of serotonin concentrations, BON cells were treated with sortilin inhibitor AF38469 (10µM, MedChemExpress) or vehicle for 24 h and cultured in serum-free DMEM/HamsF12 (Biochrom), supplemented with 0.1% BSA and 1% penicillin/streptomycin. After 24 h, the supernatant (200

µl) was removed, precipitated with the HPLC lysis buffer containing 1.68% perchloric acid and 0.1 mg/ml ascorbic acid (both from Sigma-Aldrich) in H2O, and stored at -80°C. The cell layer was harvested with 200 µl HPLC lysis buffer. After centrifugation (20,000g for 20 minutes at 4°C), the supernatants containing the cell lysates were stored at -80°C.

Murine ileal organoids were cultured and differentiated as described above and treated without or with sortilin inhibitor AF38469 (10 µM, MedChemExpress) for 96 h during EEC differentiation. For the last 24 h of the experiment, organoids were kept in serum-free DMEM/HamsF12 (Biochrom) instead of Advanced Dulbecco's Modified Eagle's Medium/F12 without HEPES and Glutamax. Afterwards, the medium was removed and the organoids dissociated into single cells by incubating in TryplE (Gibco) for 5 min at 37°C followed by washing in PBS/BSA. After centrifugation, cells were counted and transferred to the HPLC lysis buffer. After centrifugation (14,000 g for 20 min at 4°C) the supernatants were stored at -80°C.

Serotonin measurements were performed using high-sensitive reversed-phase high-performance liquid chromatography (HPLC) with fluorometric detection as previously described (13). Briefly, separation of samples was performed over a C18 reversed phase column (LipoMare C18, AppliChrom, Oranienburg, and ProntoSIL 120 C18 SH, VDS Optilab, Berlin) in a 10 mM potassium phosphate buffer (pH 5.0) (Sigma-Aldrich, Steinheim, Germany) including 5% methanol (Roth, Karlsruhe, Germany) with a flow rate of 0.8-1.0 mL/min at 20°C. The excitation wavelength was 295 nm and the fluorescent signal was measured at 345 nm. Analyses of peak parameters of chromatographic spectra and quantification of substance levels, based on comparative calculations with alternately measured external standards, were performed by using the CLASS-VP software (Shimadzu, Tokyo, Japan).

RNA isolation and quantitative reverse transcriptase PCR

Total RNA from BON cells or EEC-enriched ileal organoids was isolated with the NucleoSpin® RNA kit (Macherey-Nagel) according to the manufacturer's instructions. RNA concentration and purity were determined with a NanoDrop™ OneC (Thermo Fisher Scientific). cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad) and qRT-PCR performed using the PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific) on a QuantStudio™ 3 (Thermo Fisher Scientific). The qRT-PCR conditions were as follows: 42 cycles of 15s at 95°C, 15s at 58°C, and 30s at 72°C. Relative quantification was calculated with the ΔΔ Ct method with human hypoxanthine phosphoribosyltransferase 1 (HPRT1) and murin beta-microglobulin (ßMgi) as reference genes (Table 1).

TABLE 1 Primer sequences for gene expression analysis by qRT-PCR.

Target	fw	rv
h*TPH1*	TACTGGCGCCCGAG TTTTAG	GCACAATGGTCCAG GTCAGA
h*HPRT1*	CCCTGGCGTCGTGA TTAGTG	CGAGCAAGACGTTC AGTCCT
m*Syn*	TTGGCTTCGTGA AGGTGC	CTGCCGCACGTA GCAAAG
m*Chga*	AGAAGTGTTTGAGAAC CAGAGCCC	TTGGTGATTGGGTATTGG TGGCTG
m*ßMgi*	TTCTGGTGCTTGTCTC ACTGA	CAGTATGTTCGGCTTC CCATTC

h, human; m, murine.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's t-test when comparing pairs of means, one-way ANOVA with Bonferoni's Test when comparing more than two groups, and Spearman correlation for correlation analyses of ordinal data (*p < 0.05; ** p < 0.01; *** p < 0.001) with the GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, USA). Additionally, Welch's correction was performed to compare the means of immunoreactivity in functional vs. non-functional NETs (Figure 1).

Results

Sortilin is a marker of functional NETs

First, we performed immunohistochemical analysis of a cohort of 49 well-differentiated NET samples for sortilin expression. This cohort was derived from 41 patients (22 male and 19 female, median age 58.4 years) and included 14 intestinal and 7 pancreatic primary tumors as well as 28 hepatic metastases of 15 intestinal, 10 pancreatic, 1 lung, and 2 unknown primary tumors. Of these, 30 tumors were functional and 19 non-functional. Staining intensity was estimated by an immunoreactivity score as previously described (10). We found high sortilin immunoreactivity in a subpopulation of NETs. Expression did not correlate with tumor site, sex, or Ki67 index (Supplementary Figures 1A-E). Interestingly, sortilin expression was twice as high in tumors that caused hormone-associated disease (Figures 1A, B). To exclude that the difference was due to bias between primary tumors and metastases, we performed a subgroup analysis of primary tumors only and confirmed about two times higher expression in primary tumors of patients with functional syndrome (Figure 1C). To further exclude bias due to the tissue origin of the NETs, we re-analyzed the subgroup of small intestinal tumors and again confirmed a twofold higher sortilin expression in functional NETs. (Figure 1D). The same result was observed for subgroup analysis of liver metastases of all NETs (Figure 1E) and of liver metastases from patients with small intestinal NETs only (Figure 1F). To confirm these findings, we performed Western blot analysis of six different liver metastases of small intestinal NETs and found higher sortilin expression in functional tumors (Figure 1G). Thus, our data

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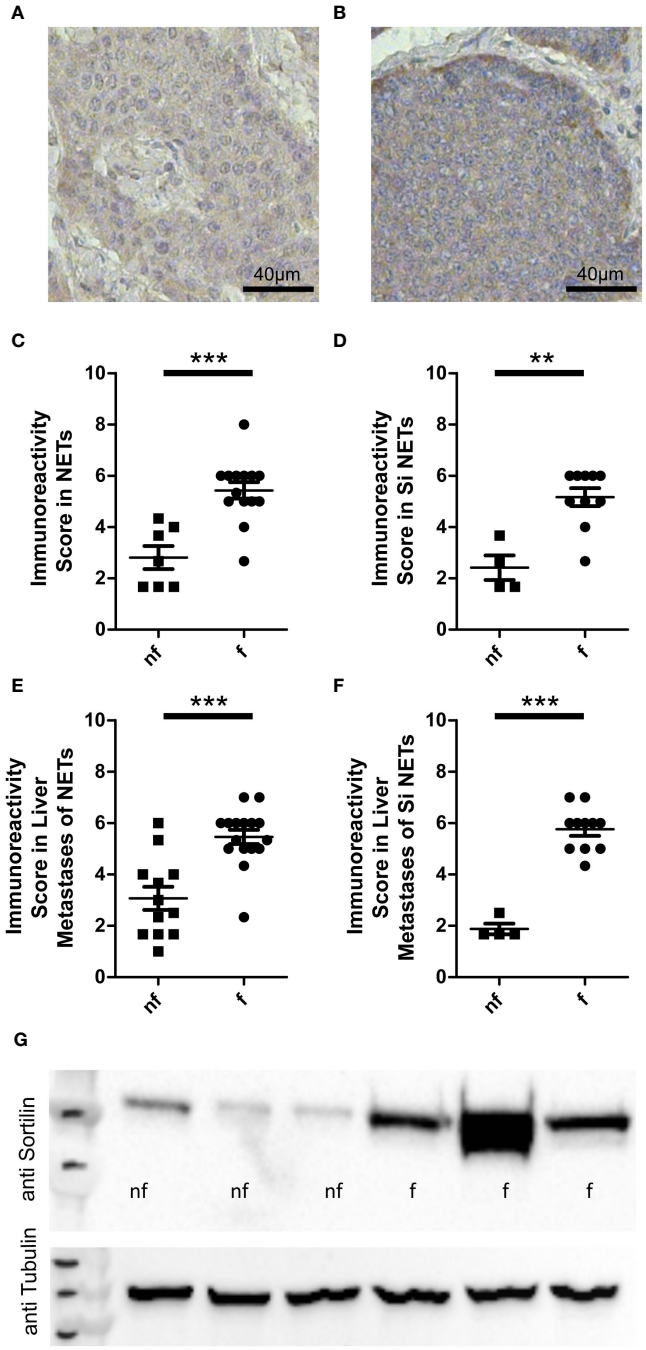

FIGURE 1
Sortilin expression in functional and non-functional neuroendocrine tumors (NETs). Neuroendocrine primary tumor tissue sections (n=21) and liver metastases of neuroendocrine tumors (n=28) were stained with an anti-sortilin antibody. Immunoreactivity scores (the product of staining intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and percentage of positive staining cells (0, no positive; 1, 0%-10% positive; 2, 11%-50% positive; 3, 51%-100% positive)) were compared between functional (f) and non-functional (nf) NETs. Representative image of a non-functional **(A)** and a functional **(B)** liver metastasis of a small intestinal NET. **(C-F)** Immunoreactivity in functional and non-functional **(C)** primary NETs (7 pancreatic NETs and 14 small intestinal NETs, 7 nf vs. 14 f) **(D)** well-differentiated small intestinal NETs (n=14, 4 nf vs. 10 f), **(E)** liver metastases of NETs (10 pancreatic, 1 lung, 15 small intestinal and 2 NETs of unknown origin, 12 nf vs. 16 f), and **(F)** liver metastases of well-differentiated small intestinal NETs (n=15, 4 nf vs. 11 f). **(G)** Sortilin expression analyzed by Western blot of 3 non-functional (nf) and 3 functional (f) liver metastases of well-differentiated small intestinal NETs. **p<0.01, ***p<0.001.

indicate that high sortilin expression is a marker of functional NETs.

We also observed a moderate correlation between sortilin expression and patient age. This effect was attributed to the lower age of patients with a functional syndrome in our cohort, as no correlation with age was found when the analysis was restricted to functional tumor samples only (Supplementary Figures 1F-H).

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Sortilin inhibition decreases serotonin production in functional NET cell cultures

To examine if sortilin is directly involved in hormone production or secretion from NETs, we next analyzed the functional consequences of sortilin inhibition. For this, we focused on one of the most common functional syndromes, carcinoid syndrome, which occurs mainly due to the overproduction and secretion of serotonin (14). As a model system, we used the serotonin-secreting neuroendocrine BON cell line (15). Western blot analysis confirmed that these cells express sortilin (Figure 2A), as reported previously (10). Next, BON cells were cultured for 24 h in serotonin-free medium with or without addition (10 µM) of the small molecule sortilin inhibitor AF38469 (16). Immunofluorescence labeling for serotonin revealed that cells treated with the inhibitor contained less serotonin than untreated control cells (Figure 2B: control, Figure 2C: 24h sortilin inhibition). To quantify the results, we measured the serotonin content in cell lysates (Figure 2D) using high-performance liquid chromatography (HPLC). Compared to control cells, the cellular serotonin content was 60% lower in cells treated with sortilin inhibitor (80.17 ± 11.58 vs. 31.5 ± 4.28 ng/ml). Reduced intracellular serotonin concentration could be caused by reduced production or increased metabolism or secretion. To rule out the latter possibility, we quantified serotonin concentrations in the cell culture medium and could not detect any amount after sortilin inhibition (Figure 2E). Furthermore, we quantified 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of serotonin, in the supernatant and found a 67% decrease of 5-HIAA levels after sortilin inhibition indicating that an increased metabolism of serotonin is not the reason for reduced cellular serotonin concentrations (Supplementary Figure 2). To rule out that the reduced serotonin production resulted from any toxic effects of sortilin inhibition, we quantified the number of live cells by using the trypan blue assay but did not observe any effects on total cell number or the percent of live cells upon AF38469 treatment (Supplementary Figures 3A, B). Furthermore, inhibition of sortilin did not affect cell proliferation, as shown by the alamar blue proliferation assay (Supplementary Figure 3C).

To corroborate impaired serotonin production in cells treated with sortilin inhibitor, we performed qPCR for tryptophan hydroxylase 1 (TPH1), the key enzyme of serotonin synthesis. We observed a significant reduction of TPH1 expression by approximately 75% after 6 h and by approximately 60% after 8 h of sortilin inhibition compared to untreated cells (Figure 2F). This effect was restored after 24 h. In summary, sortilin inhibition leads to impaired serotonin production through decreased expression of TPH1.

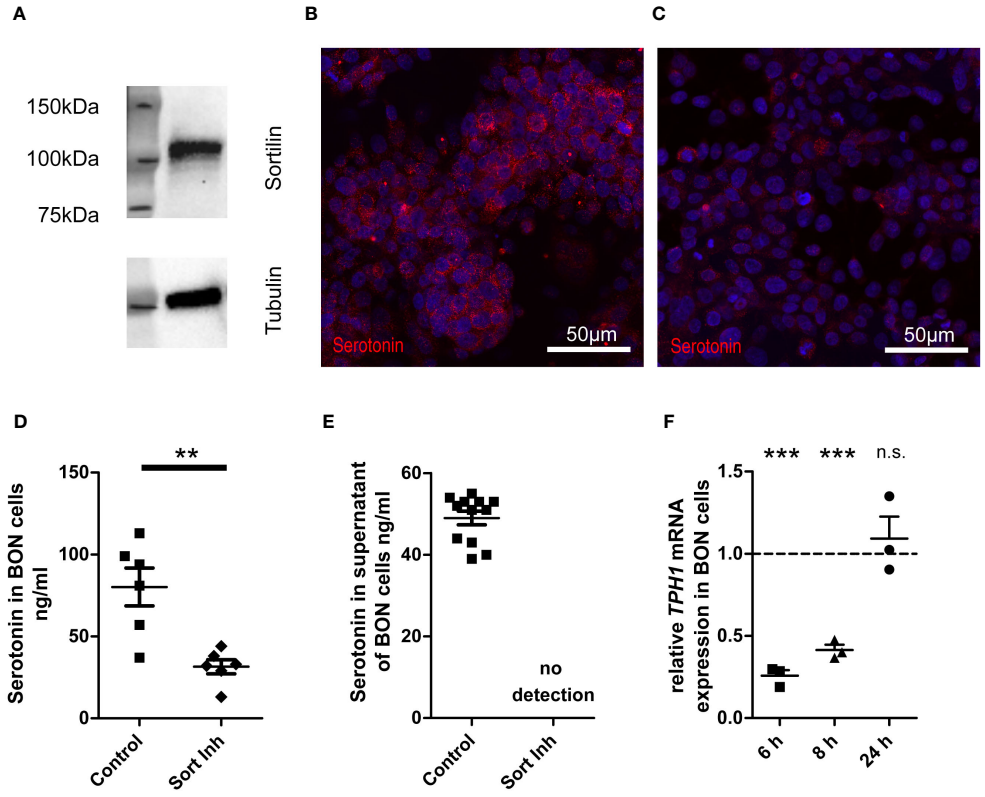

FIGURE 2
Impact of sortilin inhibition on serotonin content of NET cells. BON cells as a model of serotonin-expressing and -secreting NETs were treated for 24 h with the sortilin inhibitor AF38469 (10 µM). **(A)** Sortilin expression was analyzed by Western blot in BON cells (n=1). **(B, C)** BON cells stained for serotonin **(B)** without and **(C)** with 24 h sortilin inhibition. **(D)** Quantification of serotonin in BON cell lysates with and without 24 h sortilin inhibition (n=6 for each). **(E)** Quantification of serotonin in BON cell supernatants with and without 24 h sortilin inhibition (n=12 for each). **(F)** Relative TPH1 expression in relation to untreated BON cells 6, 8, and 24 h after addition of the sortilin inhibitor (n=3 for each). **p<0.01, ***p<0.001, n.s., not significant.

Sortilin inhibition decreases serotonin levels in differentiated enteroendocrine organoids

Although it is unknown why some NETs are functional and others non-functional, it is assumed that the basic mechanisms of hormone production and secretion in NETs are similar to those in healthy enteroendocrine cells (EECs), the suspected cell of origin of NETs (17–20). Therefore, we used murine intestinal organoids to assess the relevance of sortilin for hormone production and secretion from EECs (21). As the proportion of EECs in intestinal organoids is low, recapitulating the rare occurrence of EECs *in vivo* (0.1-1%), we applied growth factors to the culture medium to enrich organoids for EECs, as previously described (22, 23). Immunostaining for synaptophysin and qPCR for synaptophysin and chromogranin A confirmed enrichment of EECs (Figure 3A; Supplementary Figures 4A, B). EEC-enriched organoids produced sufficient amounts of serotonin to enable detection in pooled lysates (Figure 3B, control). Next, we treated EEC-enriched organoids with AF38469 for 4 days. After treatment, organoids showed a 25% lower concentration of serotonin per cell when compared to control conditions (Figure 3B, Sort Inh). This result indicates a role for sortilin in serotonin production in enteroendocrine cells. However, due to the small sample size of the treated group (n=2), additional validation of the mechanism should be performed in further studies.

Discussion

In summary, we demonstrate that functional NETs express twice as high levels of sortilin than non-functional NETs, making it the first molecular marker of NET hormonal activity. Using cell culture and EEC-enriched organoids we demonstrate that sortilin inhibition leads to reduced serotonin levels. In BON cells, this was due to reduced serotonin synthesis, most likely through lower TPH1 expression. How sortilin inhibition leads to reduced TPH1 expression remains unclear at present but may entail direct or indirect molecular mechanisms, in line with the many activities of this multifunctional receptor (24–28). Another limitation of the current study is that we observed higher levels of sortilin expression also in functional pancreatic NETs. It is important to note that the functional syndrome of pancreatic NETs is generally not caused by serotonin. Further investigation is needed to identify the way of action in this condition.

In patients with carcinoid syndrome, the overproduction and secretion of hormones, including serotonin, leads to symptoms negatively influencing quality of life and, additionally, to a tumor-independent shortening in overall survival (4, 5, 29). Besides surgery, peptide radioreceptor therapy (PRRT) and local ablative therapies, there are only limited pharmaceutical treatment options available (29). These include somatostatin analogues, the TPH1 inhibitor Telotristat, which only has a strong effect in the treatment of diarrhea (30, 31), and interferon alpha, which has a low tolerability due to side effects (29). One reason for the limited pharmaceutical treatment options is our limited understanding of functional syndrome. Even the molecular reasons why some NETs are functional and some are not remain to be identified. Here, we report sortilin as a novel target for discriminating between functional and non-functional NETs. Furthermore, sortilin is not only a marker of functional NETs, it is also directly involved in the synthesis of one of the main hormones released by functional NETs: serotonin. As sortilin inhibition diminishes serotonin production, receptor antagonists may represent a novel therapeutic strategy for treating carcinoid syndrome. Sortilin is already an established drug target for other diseases. AL001, an anti-sortilin antibody, has currently reached a phase III study for treatment of frontotemporal dementia (NCT04374136), and TH1902, a drug consisting of docetaxel conjugated to a sortilin-targeting peptide, is currently

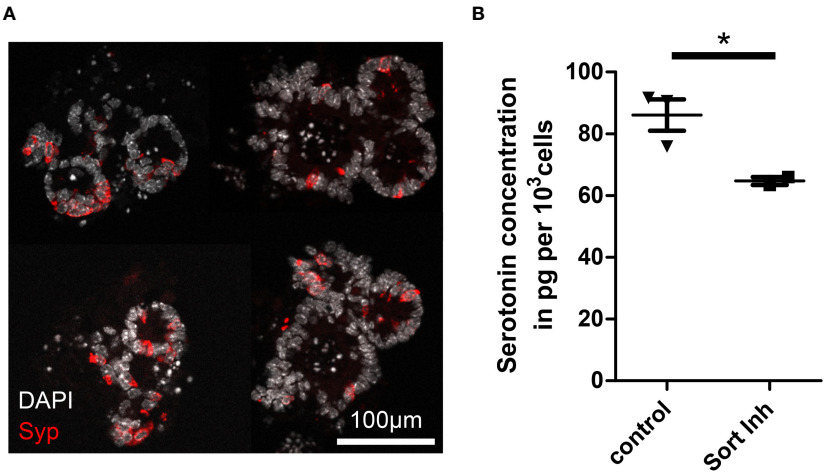

FIGURE 3
Impact of sortilin inhibition on serotonin content of enteroendocrine-differentiated organoids. **(A)** Fluorescence micrograph showing representative murine ileal organoids enriched for enteroendocrine cells (EECs). **(B)** Serotonin concentration per 1,000 cells in EEC-enriched organoids without (control) and with sortilin inhibition using AF38469 (10µM) (n=3 for control (2 biological replicates) and n=2 for sortilin inhibition (1 biological replicate), *p=0.048).

being tested in a phase I study for treatment of several solid cancers (NCT04706962). These drug candidates could be repurposed to augment the landscape of pharmaceutical treatment options for functional NETs. However, it should be noted that the current study did not evaluate AL001 and TH1902.

In order to investigate the mechanisms underlying human diseases, models are indispensable. Especially for small intestinal NETs, which cause the majority of carcinoid syndromes, appropriate models other than cell lines are lacking (22). To our knowledge, there is only one animal model of small intestinal NETs (32). However, this model on RT2 background mice only showed tumor formation in 12 out of 30 mice and only 22% out of these tumors were serotonin-positive. Although their ability to cause functional syndrome has not yet been assessed, the small proportion of serotonin-positive tumors raises doubts over the suitability of this model. Recently, attempts were made to use patient-derived organoids as a model of small intestinal NETs (33, 34), but have so far failed to model functional NETs. To our knowledge, the only functional NET organoid model described was developed by Kawasaki et al. (33), but consists of a gastrin-producing organoid line from a gallbladder NET and thus does not model carcinoid syndrome. Here, we used EEC-enriched normal intestinal organoids to explore hormone production and secretion. These organoids do indeed seem to be suitable as a surrogate model for functional small intestinal NETs, as EECs are widely accepted as the cell of origin of NETs and the mechanisms of hormone production do not differ between EECs and NETs (17–20). Our finding that sortilin inhibition causes decreased serotonin levels in these organoids underpins its role in carcinoid syndrome of functional NETs and makes it a novel potential target for treating this syndrome.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Charité - Universitätsmedizin Berlin (No EA1/229/17). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Landesamt für Gesundheit und Soziales, Berlin, T-CH0032/20. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

FB: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. AW: Investigation, Writing – review & editing. YG: Investigation, Writing – review & editing. IE: Investigation, Writing – review & editing. NA: Investigation, Writing – review & editing, Resources. MB: Formal analysis, Resources, Writing – review & editing. TW: Conceptualization, Resources, Writing – review & editing, Formal analysis. BW: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. MS: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1331231/full#supplementary-material

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EDITED BY
Bojana Popovic,
University of Belgrade, Serbia

REVIEWED BY
Madson Almeida,
University of São Paulo, Brazil
Katarzyna Ziemnicka,
Poznan University of Medical Sciences, Poland
Chunzhang Yang,
National Cancer Institute (NIH), United States

*CORRESPONDENCE
Thao Nguyen
Thao.nguyen@medicine.ufl.edu
Zehra Ordulu
mordulusahin@ufl.edu

†These authors share first authorship

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Case report: A novel somatic *SDHB* variant in a patient with bladder paraganglioma

Thao Nguyen[1*†], Zehra Ordulu[2*†], Sunaina Shrestha[2], Urja Patel[3], Paul L. Crispen[4], Lisa Brown[5], Sara M. Falzarano[2,4,6], Hans K. Ghayee[3,7] and Juan Pablo Perdomo Rodriguez[3]

[1]Department of Medicine, University of Florida, Gainesville, FL, United States, [2]Department of Pathology, Immunology and Laboratory Medicine, University of Florida College of Medicine, Gainesville, FL, United States, [3]Division of Endocrinology, University of Florida, Gainesville, FL, United States, [4]Department of Urology, University of Florida, Gainesville, FL, United States, [5]Department of Genetics, University of Florida, Gainesville, FL, United States, [6]Health Cancer Center, University of Florida, Gainesville, FL, United States, [7]Division of Endocrinology, Malcom Randall Veterans Affairs (VA) Medical Center, Gainesville, FL, United States

Background: Paragangliomas (PGL) are rare neuroendocrine tumors derived from the autonomic nervous system paraganglia. Urinary bladder paragangliomas (UBPGL) originate from the sympathetic neurons of the urinary bladder wall and represent 0.7% of all paragangliomas and <0.05% of all bladder tumors. PGL and UBPGL can be associated with *SDHB, SDHD, NF1, and VHL* gene variants, with the most common germline alterations found in *SDHB* and *VHL*.

Case report: We report a case of a 42-year-old woman who presented with menorrhagia/hematuria, uterine leiomyomas, as well as cardiac and bladder masses. The cardiac mass was favored to be a myxoma based on clinical findings, while the bladder mass was diagnosed as UBPGL. A novel *SDHB* mutation (c.642G>A, p Q214Q), detected in the UBPGL, was proven to be somatic. Although this variant was seemingly synonymous, it was predicted to have a loss of function due to the splice site effect, which was further supported by the immunohistochemical loss of *SDHB*.

Conclusion: This case highlights the challenges of diagnosing an extremely rare entity, bladder paraganglioma, with an emphasis on the multidisciplinary approach to navigate various clinical and imaging findings that may initially be misleading. In addition, a novel loss of function *SDHB* variant that could have been overlooked as a synonymous variant is herein reported, while also illustrating the importance of both germline and somatic mutation testing.

KEYWORDS

paraganglioma, bladder paraganglioma, *SDHB*, c.642G>A, p.Q214Q, leiomyoma

Introduction

Paragangliomas (PGLs) are rare neuroendocrine tumors derived from extra-adrenal chromaffin cells (1). The incidence of paragangliomas is often described together with the incidence of pheochromocytomas (PCCs), which is approximately 0.6 cases per 100,000 person-years (2). PGLs located in the neck and skull are usually parasympathetic and nonfunctional while those located in the thorax, abdomen, and pelvis tend to be sympathetic and hypersecretory (1). Urinary bladder paragangliomas (UBPGL) are extremely rare tumors originating from the sympathetic neurons of the urinary bladder wall, which represent 0.7% of all PGLs and <0.05% of all bladder tumors (3). UBPGLs may manifest with catecholamine excess, ranging from 55% to 91% of the cases (4–6) Diagnosing non-functioning UBPGLs can be challenging, as they may only manifest as hematuria or be detected incidentally from imaging studies. Consequently, up to two-thirds of UBPGLs are diagnosed following surgery or a biopsy (7).

Risk factors for metastatic UBPGLs include high levels of catecholamine excess, young age, and large tumor size (7).PGLs and UBPGLs can be associated with mutations involving *SDHB, SDHD, VHL*, and *NF1* (2). The former two genes, particularly *SDHB* are the most commonly mutated genes in patients with germline mutations (7). Previous research has illustrated that pathogenic *SDHB* variants in PGLs increase the risk of metastatic disease (7, 8).

Case presentation

A 42-year-old female with menometrorrhagia secondary to presumed uterine leiomyomas presented for hysterectomy evaluation with her gynecologist. The patient had a Foley catheter placed due to urinary retention and hematuria, for which she underwent cystoscopic examination at an outside facility. A bladder mass was identified and biopsied, revealing a paraganglioma. She was thus referred to our institution for further evaluation of the bladder mass.

A CT intravenous pyelogram revealed an enhancing 3.7 cm bladder mass centered near the left ureterovesical junction without hydronephrosis (Figure 1A), as well as a hyperdense cardiac mass on the atrial septum, incompletely evaluated. A subsequent pelvic and abdominal MRI highlighted a well-defined heterogeneous soft tissue mass in the anterior inferior aspect of the bladder abutting the urethra and vagina, measuring 3.8x3.2x3.0 cm, that demonstrated a mild hyperintense signal on T2-weighted images (Figure 1B) as well as an isointense signal on T1-weighted images. This also showed a dominant 7.4 cm intramural leiomyoma in the anterior myometrial wall with a physiologic left ovarian cyst and no other cystic solid adnexal lesions. A transurethral resection of the bladder mass (TURBT) was scheduled.

In the meanwhile, based on the initial pathology results, the patient was also referred to endocrinology. Upon further interview at the endocrinology clinic, the patient reported excessive bleeding, both during her period and between her periods (menometrorrhagia), as well as episodes of spotting between menses. Bleeding episodes varied in quantity and quality; it is possible that the hematuria could have been masked by the abundant menometrorrhagia. She also reported fatigue and shortness of breath, which was attributed to severe iron deficiency secondary to abnormal uterine bleeding. Moreover, she complained of mild abdominal pain but denied episodic headaches, sweating, tremors, palpitations, anxiety, fevers, dysuria, or recreational drug use. The patient had no other past medical history or concomitant medication except for multivitamin supplements. Her family history was significant for stroke in her grandparents and father. There was no known family history of endocrine tumors.

Initial blood pressure and heart rate were 110/80 mmHg and 70–80 beats per minute respectively. However, ambulatory blood pressure monitoring revealed occasional values of 160/90 mmHg. At night, her blood pressures did not decrease. Of note, blood pressures were not monitored post-micturition. The physical exam revealed normal heart rate and rhythm with no murmur, rub, or gallop.

Further workup showed a slightly elevated plasma metanephrine level of 74 pg/mL (normal <57 pg/mL) with normal plasma normetanephrine and serum chromogranin levels. In her 24-hour urine studies, the levels of dopamine (564 mcg/24h; normal 52–480 mcg/24h) and metanephrine (305 mcg/24h; normal 58- 203 mcg/

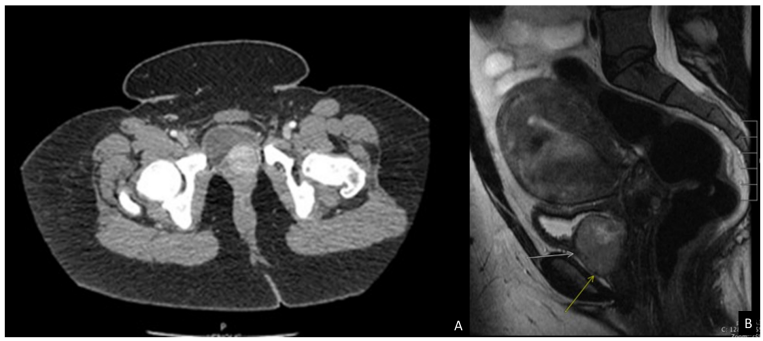

FIGURE 1
Imaging studies of the patient. **(A)** CT Intravenous pyelogram demonstrating an enhancing 3.7 cm bladder mass centered near the left ureterovesical junction without hydronephrosis or lymphadenopathy. **(B)** MRI pelvis and abdomen showing a well-defined 3.8 cm mass (arrows) in the anterior inferior aspect of the bladder abutting the urethra and vagina with a mild hypertense signal on T2 weighted image.

24h) were slightly elevated, while the normetanephrine level was normal.

There were no signs of pheochromocytoma or abdominal paragangliomas on abdominal MRI. To further evaluate synchronous paraganglioma, 68Ga-DOTATATE-PET/CT scan was performed before surgery and showed no evidence of metastatic disease, and no uptake of the hyperdense cardiac mass. Unfortunately, bladder visualization was limited due to the excreted tracer. Of note, both abdominal MRI and PET scans revealed suspicious breast nodules, however, biopsy showed fibroadenomatoid changes consistent with sclerosing adenosis with no evidence of malignancy.

To further characterize the cardiac mass, an echocardiogram was performed, which did not show the mass seen earlier on the CT scan. Further workup with cardiac MRI showed a well-circumscribed mass in the interatrial septum measuring 2.0 cm x 1.7 cm, with heterogeneous pattern in the late gadolinium enhancement sequences. A cardiologist was consulted for the left atrial mass, who concluded that it was most likely benign atrial myxoma based on MRI findings. No further treatment, including anticoagulation, was recommended.

To prevent an intraoperative hypertensive crisis, given occasional hypertension on ambulatory blood pressure monitoring, the patient received alpha blockade with doxazosin 2mg daily, which was up titrated to target blood pressure <130/80 mmHg supine and systolic blood pressure 90–110 mmHg upright, and to eliminate all occasionally high values. Her final dose before surgery was 8 mg twice daily. She then underwent TURBT for UBPGL resection. Doxazosin was continued post-operatively due to an elevated blood pressure reading. However, she decided to go home immediately after the surgery rather than stay overnight for monitoring. Therefore, she was discharged with doxazosin. Ten days later, she was seen by her cardiologist for a follow-up regarding her cardiac mass, and her dose was reduced. The patient had multiple rescheduled and missed appointments with endocrinologists. When she eventually followed up with endocrinology at three-month post-procedure, her dose was completely discontinued.

On pancystoscopy, the bladder mass appeared as a large (greater than 5 cm) endophytic mass of the left lateral and anterior walls that extended to the bladder neck. The gross specimen consisted of multiple irregularly and rectangularly shaped fragments of soft pinkish tissue that in total measured 7.0x6.0x1.0 cm in aggregate. Histologic sections showed again fragments of paraganglioma, composed of variably sized nests of eosinophilic cells with round to oval nuclei mostly arranged in a classic Zellballen pattern of growth, separated by a delicate fibrovascular network. No comedonecrosis or vascular invasion was identified. Immunohistochemical stains showed strong diffuse immunoreactivity with synaptophysin, chromogranin, and GATA-3, with negative keratin (AE1/AE3) and p63 stains (Figure 2). The ki-67 labeling index was greater than 3% (7.5% on average, as calculated over 1000 cells from hot spots in a representative section of the tumor). SDHB immunohistochemistry testing showed loss of stain in the tumor cells, with appropriately staining internal controls, represented by the endothelial and stromal cells (Figure 3). Overall, a diagnosis of primary UBPGL was made based on clinicopathologic findings.

Both germline and tumor genetic testing were undertaken as part of the clinical work-up. The germline testing was performed via the Ambry Genetics Custom-Next Cancer Panel (consisting of 85 genes) with RNA Insight and revealed a variant of unknown significance (VUS) in *MET* (c.816G>C, pQ272H). The tumor sample was sequenced by using our institution's clinically validated 700-gene next-generation sequencing (NGS) panel known as UF Health Pathlabs Gatorseq700 NGS Screening Panel to evaluate for mutations and copy number variants. Briefly, genomic DNA extracted from the tumor was amplified using the GatorSeq700 NGS Panel and sequenced on the Novaseq 6000 to high uniform depth (targeting 500x coverage by non-PCR duplicate read pairs with >99% of exons at coverage >100x). Sequence data

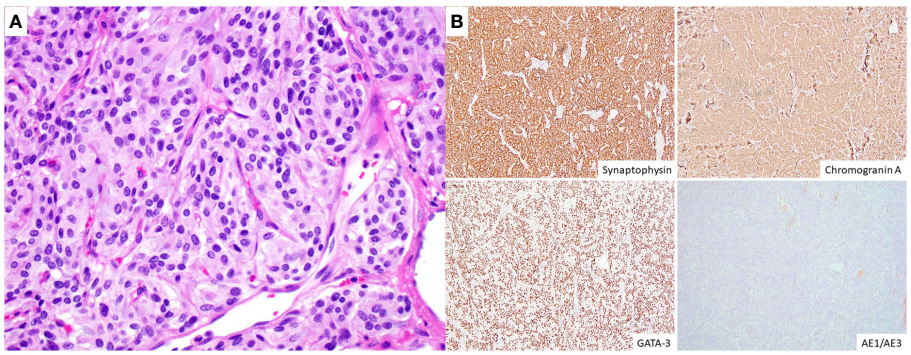

FIGURE 2
Histologic and immunohistochemical findings. **(A)** High magnification image (H&E, original magnification 400x) illustrating the tumor cells arranged in irregular nests surrounded by a fine capillary network, and showing moderate to abundant, eosinophilic cytoplasm, round to oval nuclei with finely granular ("salt and pepper") chromatin and inconspicuous to absent nucleoli. **(B)** The tumor cells (immunohistochemistry, original magnification 100x) show diffuse cytoplasmic expression of synaptophysin and chromogranin A and nuclear immunoreactivity for GATA-3, with negative keratin (AE1/AE3) stains.

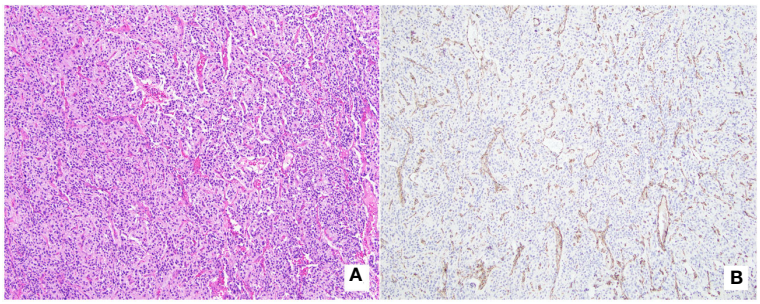

FIGURE 3
SDHB-immunochemistry with matching histology. **(A)** (H&E, original magnification 100x): Tumor is composed of cells with abundant eosinophilic cytoplasm arranged in a variable sized nested pattern, with associated thin capillary network. **(B)** (*SDHB* immunostain, original magnification 100x): Immunohistochemistry testing shows loss of *SDHB* protein expression in tumor cells, with appropriately staining internal control (endothelial cells outlining the vascular spaces, and stromal cells).

were processed using the genomic analysis application DRAGEN (enrichment version 3.9.5) with UCSC hg19-altaware as the reference genome. The mutation nomenclature was based on the convention recommended by the Human Genome Variation Society and interpretation was performed per clinical guidelines (9, 10). Tumor sequencing revealed a seemingly synonymous *SDHB* mutation (c.642G>A; p.Q214Q) with a deletion in the entire short arm of chromosome 1 spanning *SDHB* (Figure 4) and no evidence of loss of heterozygosity for the *MET* VUS. In silico analysis by multiple computational prediction tools supported the deleterious effect of the novel *SDHB* variant (c.642G>A; p.Q214Q), favoring a donor loss (see Supplementary Data).

Subsequently, the patient underwent a repeated bladder cystoscopy and an additional TURBT, which was negative for any residual tumor. Unfortunately, the patient has been lost to follow-up. We have contacted her in order to stress the importance of follow-up and to inquire about her blood pressure, menometrorrhagia, and repeated biochemical profiling.

Discussion

Herein, we present a unique UBPGL case that illustrates the importance of a multidisciplinary team approach to navigate the clinical and imaging findings of this rare entity that was diagnosed during the workup of menometrorrhagia. In addition, we report a novel loss of function variant in *SDHB* which may be challenging to interpret given that there is no change in the predicted amino acid

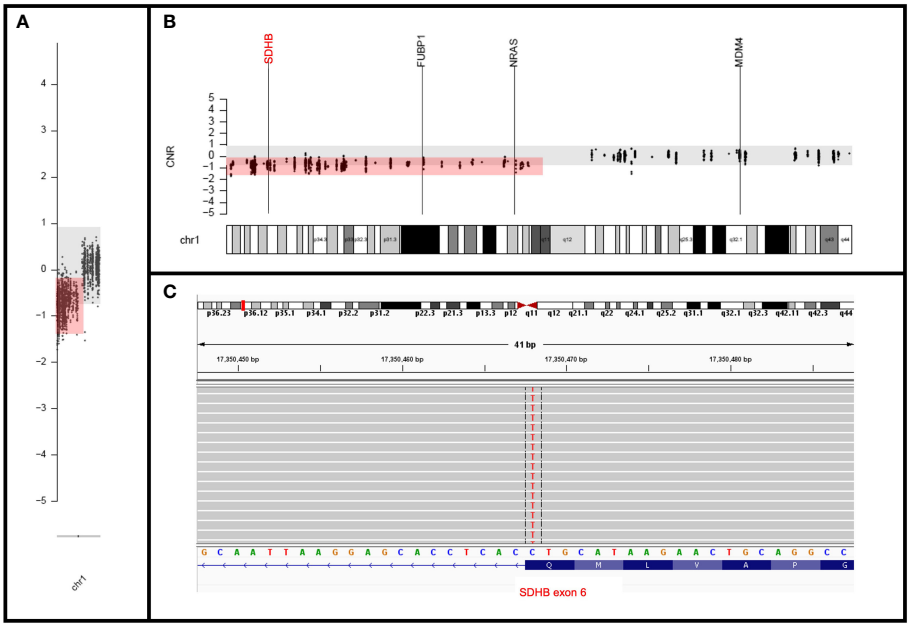

FIGURE 4
Molecular findings. **(A, B)** Copy number plots showing chromosome **(A)** and band level **(B)** loss of entire short arm of chromosome 1 with deletion of *SDHB*. **(C)** IGV view of the mutation in the last nucleotide of the *SDHB* exon 6 (negatively oriented gene) at the splice site.

transcription if one solely focuses on the nucleotide changes in the codon (c.642G>A p.Q214Q).

PCCs are rare neuroendocrine tumors deriving from chromaffin cells of the adrenal glands, and approximately 10% of these neoplasms are in the extra-adrenal sites and are referred to as PGLs. Among them, UBPGLs are one of the rarest forms with only a couple of hundred cases reported in the literature (3, 5). They can manifest with a range of symptoms, although they may remain clinically silent. Between 30–53% of UBPGLs present with symptoms of catecholamine excess triggered by micturition, 35–47% present with hematuria, while about 3–10% are discovered on imaging incidentally (4–6). Up to 45% of the UBPGLs may be non-functioning (4–6). When there is clinical suspicion for a UBPGL, evaluation includes a cystoscopy and a CT scan of the abdomen and pelvis (11). In a retrospective study by Zhang et al. (12) looking at imaging characteristics of 16 UBPGL cases (9 of which were female patients), 13 patients underwent CT scans, which all exhibited slight hypoattenuation and moderate to marked enhancement of the bladder mass. There was only one case with leiomyomas on imaging, but it was unclear if that patient was symptomatic (12). No other reported cases of concurrent leiomyomas were noted in the literature. A multicentric study that investigated 110 patients diagnosed with UBPGL showed that only 37% were diagnosed prior to biopsy based on more characteristic symptoms (7). Overall, these studies highlight that PGLs may not be considered in the differential diagnosis of bladder masses during the initial workup.

Likewise, in our patient's case, the history of leiomyomas and lack of significant symptoms of catecholamine excess made it challenging to initially consider a UBPGL in the differential diagnosis. Moreover, the slight increase in metanephrine level did not reach the level of significance (11) and could have been related to pain at the time. Considering the typical biochemical profile of paragangliomas against our findings, we concluded that our patient likely had a nonfunctioning bladder paraganglioma. Our case demonstrates that common tumors with expected symptoms, such as leiomyomas that present with abnormal uterine bleeding, may obscure findings of rare entities. The case also serves to encourage the clinicians to obtain detailed clinical histories and perform adequate work-up of incidental findings with a broad differential diagnosis.

Patients with UBPGLs present at a median age of 50 years and with a median tumor size of 2 cm (7). Hereditary PGLs manifest at an earlier age (approximately 15 years younger than average) and often present as multiple tumors (2, 7). Hereditary PGLs most commonly show germline mutations in *SDHB* and *VHL* (13, 14) while other genes such as *FH* (15) or *MET* (16) may rarely be involved. Studies have shown a strong correlation between loss of *SDHB* immunohistochemistry expression and *SDHx* mutations, with sensitivity and specificity both greater than 80% (15). Therefore, routine SDHB immunohistochemistry testing with PCCs or PGL tumors is a highly effective and rather inexpensive surrogate marker for *SDHx* mutations, thus representing a valuable screening tool for determining the necessity of germline testing in these tumors. The World Health Organization (WHO) classification of endocrine tumors considers *SDHB* immunohistochemistry "essential" in the histopathologic diagnosis of parasympathetic (most head and neck) PGLs and "desirable" in sympathetic PGLs (17). Our patient was relatively young and had multiple organ masses based on imaging. In addition, her UBPGL's pathology had a loss of *SDHB* expression. Although these findings may initially raise the potential for germline syndrome, she had only one histologically confirmed PGL from her urinary bladder. Her breast lesions were biopsy proven to be non-malignant and her cardiac tumor was favored to be a myxoma based on MRI and PET findings. The immunohistochemical loss of SDHB was accompanied by the tumor NGS testing finding of a novel *SDHB* variant (c.642G>A; p.Q214Q), however, germline testing excluded this variant as being hereditary. Of note, there was a germline *MET* variant (c.816G>C, pQ272H) which was interpreted by the reference laboratory and clinical team as a VUS (also supported by ClinVar entries (18) Variant ID: 1401743).

Overall, despite the initial findings pointing towards a hereditary syndrome, a thorough workup by multidisciplinary teams, including additional germline and somatic (tumor) molecular testing, favored this tumor to be a sporadic UBPGL with a novel somatic *SDHB* variant. Even in cases of *SDH*-deficient neoplasia where no germline mutation was found, surveillance and further follow-up for other *SDH*-deficient neoplasms are still recommended (19). Even though, a germline pathogenic variant was not found in our patient, future follow-up with 68Ga-DOTATATE-PET/CT will be pursued, to continue to monitor for metastases and other neoplasms, based on other risk factors present in this case, including the young (41 years) age at presentation, the relatively large UBPGL size (3.8 cm on imaging), and the tumorigenic mutation in *SDHB*.

The *SDHB* (c.642G>A; p.Q214Q) variant itself raised a molecular diagnostic challenge in this case due to its seemingly synonymous change in the protein nomenclature. However, it occurred at the last nucleotide of exon 6 (Figure 4), which was predicted to result in a splice site effect (20) in this gene with loss of function variants considered pathogenic. This amino acid position is highly conserved across species and this variant has not been observed in population databases. Different mutations at the same nucleotide position have been reported as likely pathogenic; these include c.642G>T (2 ClinVar entries, Variation ID: 480788) and c.642G>C (reported in a patient with a malignant paraganglioma) (21) and listed in Human Gene Mutation Database (CM065460). In addition, the deleterious effect of this variant was further supported by in silico analysis by multiple prediction tools (see Supplementary Material). Lastly, the WHO for Genetic Tumour Syndromes recommends performing *SDHB* immunohistochemistry for evaluation of a VUS in *SDHB*, with a loss implying pathogenicity (19).

In combination with the patient's tumor histology and the loss of immunohistochemical expression of *SDHB*, this somatic variant is ultimately interpreted as a likely pathogenic somatic variant (confirmed by germline testing) and the potential driver of this presumed sporadic tumor. Of note, there was a loss of heterozygosity of *SDHB* by deletion of the entire short arm of chromosome 1, which is a common mechanism for biallelic

inactivation in the setting of somatic mutations, first described in this tumor type by van Nederveen et al. (22).

Surgical intervention for UBPGLs is typically personalized to the patient due to the lack of prospective research and clear criteria for malignancy prediction. It is known that there are no absolute histologic criteria or single biomarkers to reliably predict the biological behavior of PGLs or PCCs, and multi-parameter scoring systems have been proposed. The GAPP (Grading system for Adrenal Pheochromocytoma and Paraganglioma) (23) and modified GAPP (24) have shown predictive value to varying degrees, and they may be particularly useful to "rule out" potentially aggressive behavior rather than "rule it in" for risk stratification. The current patient's tumor characteristics would add up to a GAPP score of 4 ("intermediate risk"). However, the risk stratification also depends on extra-adrenal location, patient age, number of tumors, and evidence of metastasis. A comprehensive approach should include clinical, biochemical, molecular, and pathological assessments (25) Short-term safety for procedures like transurethral resection and cystectomies is documented, yet long-term outcome data remains limited. Usually, UBPGLs are initially treated with either cystectomy or TURBT (7). Repeated surgery is sometimes required, especially in those of younger age (<5 years old) and large tumor size (>1 cm). Patients with incomplete resection or higher tumor stages (≥ T3) are at higher risk of recurrence, metastases, and death when compared to those with lower stages (26). Although the prognosis is usually good, about 8% of patients present with synchronous metastases, and 22% of patients develop metachronous metastases. These patients tend to be young, have a large UBPGL size, and have a high degree of catecholamine excess (4). Our patient was asymptomatic with negative repeated bladder resection at 3 months; however, our study is limited due to a lack of long-term follow-up.

This unique case of a UBPGL showcases multiple layers of diagnostic challenges starting with the patient's initial abnormal uterine bleeding and leiomyoma masking the symptoms of this rare entity. The patient's relatively young age, additional findings throughout the clinical workup of other masses at different sites, and the loss of *SDHB* immunohistochemistry in the UBPGL raised the potential for a germline syndrome, which was then excluded with germline and tumor genetic testing. Lastly, the somatic *SDHB* variant highlights the importance of positional effects at splice sites in "seemingly synonymous" variants in molecular diagnostics. Overall, a comprehensive approach combining multiple layers of data, from clinical history to molecular findings with multidisciplinary teamwork, is essential for diagnosing rare and challenging cases like ours.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

TN: Writing – original draft, Writing – review & editing. ZO: Writing – review & editing, Investigation, Supervision, Formal Analysis, Conceptualization. SS: Writing – review & editing. UP: Investigation, Writing – review & editing. PC: Investigation, Writing – review & editing. LB: Investigation, Writing – review & editing. SF: Investigation, Writing – review & editing, Resources. HG: Conceptualization, Investigation, Supervision, Validation, Writing – review & editing, Project administration, Resources. JP: Investigation, Supervision, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1386285/full#supplementary-material

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EDITED BY
Francesca Spada,
European Institute of Oncology (IEO), Italy

REVIEWED BY
Yasutaka Takeda,
Kanazawa Medical University, Japan
Bojana Popovic,
University of Belgrade, Serbia

*CORRESPONDENCE
Mariana P. Monteiro
mpmonteiro@icbas.up.pt

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

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Non-functional alpha-cell hyperplasia with glucagon-producing NET: a case report

Catarina Cidade-Rodrigues[1†], Ana Paula Santos[2,3†], Raquel Calheiros[2], Sara Santos[2], Catarina Matos[4], Ana Paula Moreira[5], Isabel Inácio[2], Pedro Souteiro[2], Joana Oliveira[2], Manuel Jácome[6], Sofia S. Pereira[7,8], Rui Henrique[3,6,9], Isabel Torres[2‡] and Mariana P. Monteiro[7,8*‡]

[1]Hospital Padre Américo, Unidade Local de Saúde do Tâmega e Sousa, Penafiel, Portugal, [2]Department of Endocrinology, Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal, [3]Research Center of IPO Porto (CI-IPOP), RISE@CI-IPO (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto), Porto Comprehensive Cancer Centre (P.CCC), Porto, Portugal, [4]Hospital de Braga, Unidade Local de Saúde de Braga, Braga, Portugal, [5]Institute for Nuclear Sciences Applied to Health (ICNAS), University of Coimbra, Coimbra, Portugal, [6]Department of Pathology, Portuguese Oncology Institute of Porto (IPO Porto), Porto, Portugal, [7]Unit for Multidisciplinary Research in Biomedicine (UMIB), School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal, [8]Laboratory of Integrative and Translocation Research in Population Health (ITR), Porto, Portugal, [9]Department of Pathology and Molecular Immunology, School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal

Introduction: Alpha-cell hyperplasia (ACH) is a rare pancreatic endocrine condition. Three types of ACH have been described: functional or nonglucagonoma hyperglucagonemic glucagonoma syndrome, reactive or secondary to defective glucagon signaling, and non-functional. Few cases of ACH with concomitant pancreatic neuroendocrine tumors (pNETs) have been reported and its etiology remains poorly understood. A case report of non-functional ACH with glucagon-producing NET is herein presented.

Case report: A 72-year-old male was referred to our institution for a 2 cm single pNET incidentally found during imaging for acute cholecystitis. The patient's past medical history included type 2 diabetes (T2D) diagnosed 12 years earlier, for which he was prescribed metformin, dapagliflozin, and semaglutide. The pNET was clinically and biochemically non-functioning, apart from mildly elevated glucagon 217 pg/ml (<209), and ^{68}Ga-SSTR PET/CT positive uptake was only found at the pancreatic tail (SUVmax 11.45). The patient underwent a caudal pancreatectomy and the post-operative ^{68}Ga-SSTR PET/CT was negative. A multifocal well-differentiated NET G1, pT1N0M0R0 (mf) strongly staining for glucagon on a background neuroendocrine alpha-cell hyperplasia with some degree of acinar fibrosis was identified on pathology analysis.

Discussion and conclusion: This case reports the incidental finding of a clinically non-functioning pNET in a patient with T2D and elevated glucagon levels, unexpectedly diagnosed as glucagon-producing NET and ACH. A high level of

suspicion was required to conduct the glucagon immunostaining, which is not part of the pathology routine for a clinically non-functioning pNET, and was key for the diagnosis that otherwise would have been missed. This case highlights the need to consider the diagnosis of glucagon-producing pNET on an ACH background even in the absence of glucagonoma syndrome.

KEYWORDS

alpha-cell, hyperplasia, neuroendocrine tumors, glucagon-producing NET, pancreas

1 Introduction

Pancreatic endocrine cell hyperplasia may occur in approximately 0.6% of adults (1), while isolated alpha-cell hyperplasia (ACH), a condition that was first reported in 1991, is even more rare (2). ACH etiology remains poorly understood (2), but has been described to affect both women and men with an age at diagnosis ranging from 25 to 74 years old (3).

Three types of ACH have thus far been described: functional, reactive, and non-functional (3).

Functional ACH represents 17% of cases (3) and presents with nonglucagonoma hyperglucagonemic glucagonoma syndrome. Therefore, glucagon levels are elevated, but no gross pancreatic neuroendocrine tumor (pNET) is found (4, 5).

Reactive ACH is the most frequent type, accounting for 42% of cases and results from defective glucagon receptor signaling, either due to inactivating mutations or deletions of the glucagon receptor gene or glucagon receptor intracellular pathways, also known as Mahvash disease. It may present with nonspecific symptoms including weight loss, abdominal pain or altered intestinal transit, or as an incidentally discovered pancreatic mass. This ACH subtype is characterized by marked hyperglucagonemia without glucagonoma syndrome, as well as the development of gross pNETs (3).

Non-functional ACH is responsible for 25% of cases (3) and is characterized by normal or slightly elevated glucagon levels. It may also present with nonspecific symptoms, although glucagonoma syndrome is usually absent. ACH is most often an incidental histological finding and its clinical relevance is the putative increased risk for pNETs.

Only a few cases of ACH associated with pNETs have been described in the literature. Among these, some reported the association of reactive ACH with glucagonoma (4, 6, 7) or a non-functioning pNET (8).

A case report of the incidental diagnosis of a non-functional ACH with a glucagon-producing NET is herein presented.

2 Case description

We present a 72-year-old male individual, referred to our tertiary reference center for a single 2 cm pNET incidentally discovered in a CT scan performed during an investigation for acute cholecystitis. The tumor was clinically non-functioning as symptoms or signs of hormone hypersecretion were absent.

The patient's past medical history included type 2 diabetes (T2D) diagnosed 12 years earlier, arterial hypertension, and dyslipidemia. T2D glycemic control over the years was overall good, with HbA1c levels ranging between 5 and 6%, while prescribed metformin 1000 mg bid and dapagliflozin 10 mg od. Furthermore, he had received treatment with once-weekly semaglutide 1mg for 3 months, 1 year before surgery. The patient had no past medical history of pancreatitis. Family history was irrelevant.

The patient's body mass index was 25.6 kg/m^2, but physical examination was otherwise unremarkable, without palpable abdominal masses or skin lesions.

Biochemical analysis showed an HbA1c 6%, insulin 5.15 uUI/ml (2.6-24.9), somatostatin < 3.9 pmol/l (< 16.0), gastrin 12.8 pg/ml (< 108), vasoactive intestinal peptide 15.7 pmol/l (< 30) and mildly elevated glucagon [217 pg/ml (<209)], as presented in Table 1. Glucagon levels were measured using a radioimmunoassay (RB310, DIAsource ImmunoAssays, S.A., Belgium), which exhibited a cross-reactivity of less than 0.1% with glucagon-related peptides.

Abdominal magnetic resonance imaging (MRI) showed a nodule in the tail of the pancreas measuring approximately 25mm in its longest axis. It was difficult to delineate, showing a hyposignal on T2-weighted images and isosignal on T1-weighted images, with increased enhancement in relation to the surrounding pancreatic parenchyma. This was in favor of the hypothesis of a neuroendocrine tumor. ^{68}Ga-SSTR PET/CT showed positive uptake in the pancreatic tail (SUV max 11.45), as shown in Figure 1. The patient underwent a caudal pancreatectomy with curative intent, which occurred without complications, and post-operative CT and ^{68}Ga-SSTR PET/CT were negative, as shown in Figure 1.

On macroscopical examination, an area with a nodular outline was identified, with a slightly increased consistency, a yellowish-white color, and imprecise boundaries. The 25mm nodule disclosed on MRI largely corresponded to chronic pancreatitis lesions. The largest tumor focus identified microscopically had 0.4cm in at the greatest diameter and corresponded to a multifocal well-differentiated neuroendocrine tumor G1 pT1N0M0R0 (mf), strongly staining for

TABLE 1 Initial blood test evaluating tumor secretion status.

Serum Parameter	Result (reference range)
Glucose	86 mg/dL (< 100)
Creatinine	0.6 mg/dL (0.7-1.3)
Aspartate aminotransferase	14 U/L (8-33)
Alanine transaminase	15 U/L (7-56)
HbA1c	5.4% (< 5.7)
TSH	5.31 µUI/mL (0.4-5.5)
Chromogranin A	41 ng/mL (< 98.1)
Glucagon	217 pg/mL (< 209)
Insulin	5.15 uUI/mL (2.6-24.9)
C-peptide	2.45 ng/mL (1.1-5.0)
Somatostatin	< 3.9 pmol/L (< 16.0)
Gastrin	12.8 pg/mL (< 108)
VIP	15.7 pmol/L (< 30)
ACTH	38.5 pg/mL (7.2-63.3)
Ionized calcium	1.26 mmol/L (1.17-1.38)
IGF-1	66.2 ng/mL (61-186)
PTH	28.9 pg/mL (12-65)
Prolactin	7.36 ng/mL (< 20)

glucagon. The pancreas presented multiple and scattered foci of fibrosis and atrophy of the exocrine pancreas, associated with neuroendocrine hyperplasia. Mitotic figures were scarce (<1 per 2mm^2) and Ki67 labeling was less than 3%. Lymphovascular or perineural invasion was not found. On immunohistochemistry, neoplastic cells disclosed immunoreactivity for cytokeratin 8/18, chromogranin, synaptophysin, and glucagon. Pancreatic islets presented a glucagon-producing α-cells percentage superior to 80%, while the percentage in normal human islets is usually under 50% even considering the larger relative proportion observed in the pancreas of individuals with T2D (9). These findings were compatible with the pathological diagnosis of glucagon-producing NET and ACH (Figure 2).

No specific genetic analysis for hereditary cancer syndromes was performed given the negative family history.

The patient remains recurrence- and symptom-free at 9 months of follow-up after surgery.

3 Discussion

This case reports the incidental finding of a glucagon-producing NET in the absence of the typical glucagonoma syndrome on a background of ACH.

ACH has been thought to be a potential preneoplastic condition, in particular the reactive and non-functional subtypes, while it remains to be proven whether the same applies to the functional subtype. Furthermore, ACH is characterized by a diffuse and specific increase in alpha-cell mass on histological analysis, which differs from the nonspecific alpha- and beta-cell hyperplasia found in patients with multiple endocrine neoplasia type 1 and von Hippel-Lindau syndrome (10–12).

The most well-characterized type is reactive ACH, since there is an identified molecular defect in the glucagon signaling pathway that can be replicated in an animal model (13, 14). In GCGR -/- mice, the progression from pancreatic endocrine cell mass

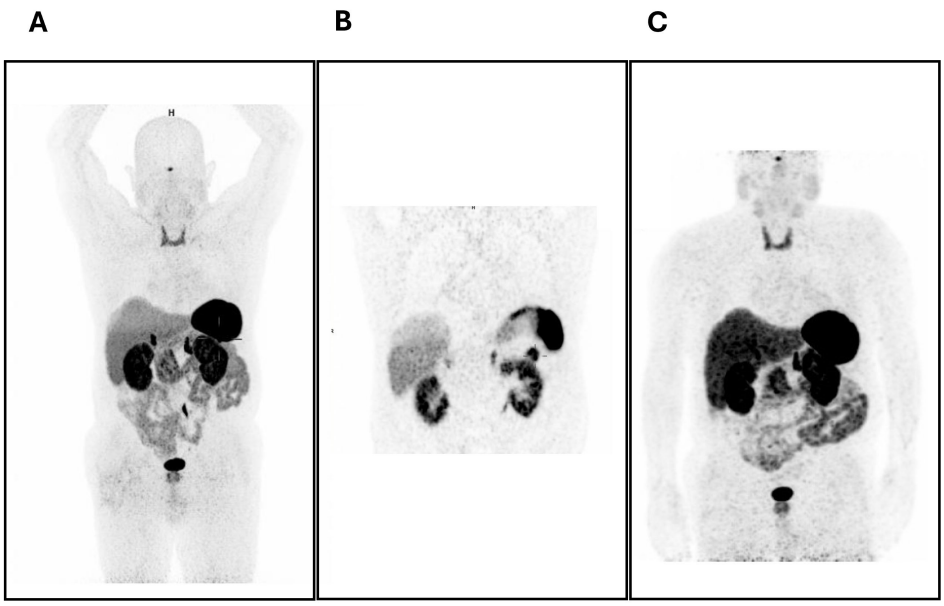

FIGURE 1
(A, B) 68Ga-DOTA-NOC PET/CT scan before treatment showing the positive uptake in the pancreatic tail (SUV max 11.45). **(C)** 68Ga-DOTA-NOC PET/CT scan after surgery showing no hyperfixation foci.

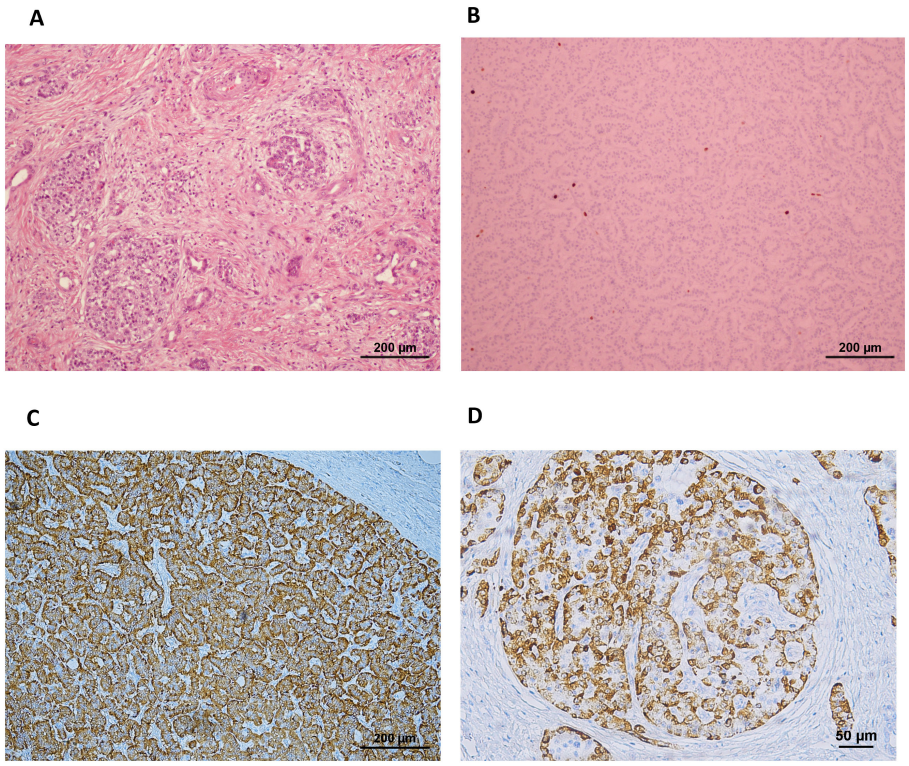

FIGURE 2
(A) H&E staining showing the pancreas with multiple scattered foci of fibrosis and **(B)** neuroendocrine cell hyperplasia including a pNET with low Ki67 labeling. **(C)** The pNET was strongly positive for staining for glucagon (ref. 565860, BD Pharmingen) on immunohistochemistry, and **(D)** the pancreatic islets depicted over 80% of the cells staining positive for glucagon, while the percentage in normal human islets is usually under 50% even considering the larger relative proportion observed in pancreas from individuals with T2D (9).

expansion with ACH to dysplasia at 5 to 7 months, proceeding to pNETs < 1 mm at 12 months and then to pNETs > 1 mm at 18 months is well-established (13, 14). Moreover, the majority of these pancreatic lesions express glucagon, although some may also express insulin or no hormones (13, 14). In contrast, the pathogenesis of functional and nonfunctional ACH, for which there are no animal models that could speed the process of unraveling the mechanisms of disease, remains obscure. Nevertheless, besides reactive ACH and at least for nonfunctional ACH, the possible contribution of hyperplastic alpha-cells and their morphological changes for the development of pNETs remains a reasonable hypothesis.

There are no specific treatment recommendations for reactive or nonfunctional ACH currently established (13, 14). Therefore, for pNETs arising within the framework of reactive and nonfunctional ACH, treatment follows the same recommendations as for sporadic pNETs occurring outside the ACH context (15). Observational surveillance could be considered for nonfunctioning tumors ≤ 2 cm. Surgical resection is the preferred treatment for non-metastatic tumors larger than 2 cm, whether these are functioning or nonfunctioning. Subtotal or total pancreatectomy has been advocated as a treatment approach for functional ACH (3).

For the patient herein described, surveillance could have been an option given that the pNET was apparently non-functioning with a size of 25 mm. However, taking into account the patient's preference and given the borderline tumor size and easy surgical access (caudal), the multidisciplinary team's decision was in favor of distal pancreatectomy surgery. Otherwise, ACH would not have been detected nor would the glucagon-producing NET have been diagnosed.

Given the concurrent diagnosis of diabetes, two questions emerge: could diabetes be a manifestation of subclinical hyperglucagonemia? Alternatively, could ACH have been elicited by the use of incretin-based anti-diabetes medications, namely glucagon-like peptide-1 receptor agonists (GLP-1Ra), subsequently contributing to the development of glucagon-producing NET?

Regarding the first question, diabetes possibly being secondary to glucagon excess is a reasonable hypothesis since glucagon seems to be more critical for the development or worsening of diabetes than insulin deficiency (16–18). Glucagon is able to induce liver glycogenolysis and raise blood glucose levels through specific binding to its receptor (GCGR) (18, 19). Indeed, the mild and subclinical hyperglucagonemia to which the patient was exposed could provide an explanation for the good glycemic control observed over more than a decade. Typical glucagonoma syndrome includes diabetes, necrolytic migratory erythema, depression, and deep vein thrombosis in the presence of hypoaminoacidemia and high glucagon levels, usually > 1000pg/ml (20–22). In fact, glucagon-producing NETs are usually larger

than 4 or 5 cm in diameter at diagnosis and, at that stage, are often associated with locoregional or even distant metastases at diagnosis (20, 21). This notwithstanding, some patients recall the presence of a few selected nonspecific features for many years before clinical deterioration coincident with the development of the classic glucagonoma constellation (23). In the herein reported case, since there was only a mild hyperglucagonemia without typical glucagonoma syndrome features, a non-functional pNET with unrelated concomitant hyperglucagonemia cannot be excluded. Although the cause for the hyperglucagonemia remains otherwise undisclosed given that the patient's past medical history was unremarkable for pancreatitis, liver, or renal impairment.

Regarding the second question, whether GLP-1Ra could have elicited ACH, this is even less likely. Indeed, GLP-1Ra are recognized to reduce glucagon levels by 20% to 50% through a strong and conserved negative feedback mechanism and consequently a reactive alpha-cell hyperplasia occurs (24). However, there is no evidence of any association between this histological finding and the risk of pNET development. Additionally, although *in vitro* and *in vivo* animal studies native GLP-1 was shown to promote pancreatic beta-cell proliferation, inhibition of its apoptosis, and differentiation of stem cells in the ductal epithelium through neogenesis in the islets (25), the same was not demonstrated to occur in humans, nor is it supported by the extensive data derived from clinical trials across the GLP-1Ra drug class (26, 27). Nonetheless, the putative long-term risk for exocrine and endocrine pancreatic neoplasia associated with incretin-based drugs has been a matter of concern, although highly controversial since the available evidence is scarce and mostly derived from animal studies or small human case series involving exenatide (25, 28, 29) and irrespective of the presence of ACH. Therefore, the hypothesis that GLP-1Ra might accelerate the progression of pancreatic dysplastic lesions, especially in the context of pre-existing chronic pancreatitis, a known predisposing factor for exocrine pancreatic cancer, and premalignant pancreatic intraepithelial neoplasia (28, 30), cannot be entirely excluded (31). However, there is no available evidence that GLP-1Ra could trigger glucagon-producing or other non-functioning pNETs.

In human *pancreata* obtained at autopsy, Butler A et al. found that individuals with T2D treated with incretin bases therapies (exenatide and sitagliptin) exhibited a 40% increase in pancreatic mass when compared to those with T2D not treated with these drugs. The authors also found higher rates of exocrine and endocrine cell proliferation and a higher prevalence of pancreatic intraepithelial neoplasia and ACH. Furthermore, among the eight individuals studied, three presented with adenomas smaller than 1 cm and 1 had an adenoma ≥ 1 cm, the majority of which stained positive for glucagon (29). It is important to note that this study was a small case series, which included only one patient treated with the GLP-1Ra exenatide (29).

Incretin-based therapies are also a cause of concern for increased risk of exocrine pancreatic neoplasms. In patients treated with GLP-1Ra, acute and chronic pancreatitis, whether clinical or subclinical, seems to ensue from duct cell proliferation and obstruction, as a predisposing factor to exocrine pancreatic cancer (25). As such and given the fact that incretin-based therapies were first introduced for T2D treatment in 2005, there is still no data regarding the true impact of these drugs in the risk for these types of tumors, since the effect present is probably too small to be detected. It should be stressed that up to this date, only exenatide has been associated with an increased risk of pancreatic cancer. Moreover, a large number of randomized clinical trials, as well as real-world data, are reassuring regarding the pancreatic safety of the GLP-1Ra drug class (32–35).

Moreover, as the frequency of ACH case reports is increasing, one might question whether the chronic use of such therapies over long periods could be involved in the upsurge of the condition (36).

As a matter of fact, pNETs appearing in the context of reactive ACH, both in humans and animals, are slow-growing tumors, often measuring only a few centimeters at the time of diagnosis. These commonly manifest later in life, particularly in middle-aged patients who may have had ACH for many years prior to diagnosis (37, 38). The surge of a glucagon-producing NET in the absence of the typical glucagonoma syndrome triad on an ACH background further supports the hypothesis that ACH could be a precursor of pNET.

4 Conclusions

The incidental finding of a clinically non-functioning pNET in a patient with T2D, unexpectedly diagnosed as a glucagon-producing NET on an ACH background, is herein reported. The concomitant presence of T2D and glucagon levels above normal, despite the absence of a typical glucagonoma syndrome triad, were the key for performing glucagon immunostaining in a clinically non-functioning pNET, without which the diagnosis of glucagon-producing NET would easily have been missed. This case highlights the need to consider the diagnosis of glucagon-producing NET on an ACH background, even in the absence of glucagonoma syndrome. Moreover, this case report reinforces the need to further explore the hypothesis of ACH as a potential precursor of glucagon-secreting pNETs or even glucagonomas.

5 Patient perspective

The patient took part in the decision process of opting for surgical removal of the 25 mm pNET that, given the small size, could have been kept under surveillance and was pleased to have made that informed choice after understanding the diagnosis.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

CC-R: Formal analysis, Data curation, Investigation, Writing – original draft. APS: Writing – original draft, Data curation, Formal analysis, Investigation. RC: Investigation, Writing – review & editing. SS: Writing – review & editing, Investigation. CM: Writing – review & editing, Investigation. APM: Writing – review & editing, Investigation. II: Writing – review & editing, Investigation. PS: Writing – review & editing, Investigation. JO: Writing – review & editing, Investigation. MJ: Writing – review & editing, Investigation. SSP: Writing – review & editing, Formal analysis, Investigation, Methodology. RH: Writing – review & editing, Data curation, Investigation. IT: Investigation, Writing – review & editing. MPM: Formal analysis, Funding acquisition, Methodology, Writing – review & editing.

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Conflict of interest

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

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EDITED BY
Bojana Popovic,
University of Belgrade, Serbia

REVIEWED BY
Giulia Puliani,
IRCCS Regina Elena National Cancer Institute, Italy
Peter Igaz,
Semmelweis University, Hungary

*CORRESPONDENCE
Miklós Bodor
mbodor@gmail.com

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Single center experience in localization of insulinoma by selective intraarterial calcium stimulation angiography - a case series of 15 years

Sándor Halmi[1,2], Eszter Berta[1,3], Ágnes Diószegi[4], Lívia Sira[1], Péter Fülöp[4], Endre V. Nagy[1], Ferenc Győry[5], Zsolt Kanyári[5], Judit Tóth[6], Harjit Pal Bhattoa[7] and Miklós Bodor[1,3]*

[1]Division of Endocrinology, Department of Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, [2]Doctoral School of Health Sciences, University of Debrecen, Debrecen, Hungary, [3]Department of Clinical Basics, Faculty of Pharmacy, University of Debrecen, Debrecen, Hungary, [4]Division of Metabolism, Department of Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, [5]Department of Surgery, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, [6]Division of Radiology and Imaging Science, Department of Medical Imaging, Faculty of Medicine, University of Debrecen, Debrecen, Hungary, [7]Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Background: Insulinomas are rare insulin-secreting neuroendocrine neoplasms of the pancreas. First-line treatment is the surgical removal of the tumor, however, the localization with standard imaging techniques is often challenging. With the help of selective intraarterial calcium stimulation the insulinoma's localization can be narrowed down to one third of the pancreas which the selected artery supplies.

Objective: We aimed to prove the usefulness of the calcium stimulation test in case of 9 patients treated between 2006 and 2021 diagnosed with endogenous hyperinsulinemic hypoglycemia confirmed by fasting test, where conventional imaging methods, like transabdominal ultrasound, CT or MRI failed to detect the source of hyperinsulinemia.

Methods: We performed selective intraarterial calcium stimulation with angiography with calcium gluconate injected to the main supporting arteries of the pancreas (splenic, superior mesenteric and gastroduodenal arteries); blood samples were obtained from the right hepatic vein before, and 30, 60 and 120 seconds after calcium administration.

Results: With selective angiography we found a significant elevation of insulin levels taken from the right hepatic vein in five of the nine cases. On histopathology, the lesions were between 1-2 cm, in one case malignancy was also confirmed. In four patients we found a significant rise of insulin levels obtained from all catheterized sites, which confirmed the diagnosis of nesidioblastosis. In three cases no surgery was performed, and the symptoms relieved with medical treatment.

Conclusions: Selective intraarterial calcium stimulation remains an important tool in localization of the source of insulin excess, especially in cases where other diagnostic modalities fail.

KEYWORDS

insulinoma, selective intraarterial calcium stimulation, ASVS, nesidioblastosis, hyperinsulinemic hypoglycemia, pancreas, functioning neuroendocrine tumor

1 Introduction

Insulinoma is a rare neoplasm of the pancreatic beta cells with an estimated incidence of 1-4/1 million (1, 2). Despite its rare occurrence, insulinoma is the most common functioning neuroendocrine tumor of the pancreas. Most insulinomas are sporadic, however, 5-10% of the cases can also present as part of multiple endocrine neoplasia type 1 and 4, or rarely neurofibromatosis type 1 or tuberous sclerosis (3). The cases with underlying endocrine tumor syndromes need even more stringent medical attention as 16-25% of the MEN-1 syndrome cases are malignant (4, 5).

Sporadic insulinomas develop mainly in middle-aged patients, but they can occur at any age with a female predominance (60%); 87-94% of insulinoma cases are benign and solitaire (2, 3, 6).

The appropriate diagnosis of insulinoma is often delayed and is established only years after the first appearance of the clinical symptoms (7, 8). The average time until biochemically verified diagnosis is at least 2 years, but can often take 5 or more years, or even longer, and repeated hypoglycemic episodes can lead to damage of autonomic nervous system (7, 9, 10). Weight gain is also a common finding occurring in 39-50% of patients (7, 8, 11).

The biochemical diagnosis of endogenous hyperinsulinemic hypoglycemia must be obtained. The primary suspicion for insulinoma should raise when Whipple's triad is present (7, 12). The suspected diagnosis based on the presence of Whipple's triad needs to be verified with a method successfully detecting 99% of the cases, namely the up to 72 hours long fasting test with concurrent measurements of beta-cell polypeptides (insulin >4 μU/mL, C-peptide >0.2 nmol/L and proinsulin >5 pmol/L) at the time of hypoglycemia (3, 7).

In most cases surgical removal of the tumor is curative. However, the localization of the tumor can be quite challenging with the widely accessible conventional imaging methods as insulinomas are usually smaller than 2 centimeters in diameter (13, 14). In patients with MEN-1 syndrome insulinomas are between 1-3 cm-s and can be multifocal (14).

According to the equal distribution of beta-cells in the pancreas, insulinomas can develop anywhere within the organ, while extrapancreatic tumors are very rare (<2%) (14–16).

One goal during surgery is to reduce the exocrine and endocrine functional loss of the pancreas; parenchyma-sparing partial pancreatectomy or tumor enucleation can only be performed after proper localization of the insulinoma. Conventional non-invasive imaging techniques like transabdominal ultrasound, contrast-enhanced CT and MRI can localize the tumor properly with detection rates of 9-63%, 63-94% and 60-90%, respectively (3). In some cases, none of the above-mentioned procedures can localize the tumor and there is need for additional diagnostic procedures. Somatostatin receptor scintigraphy has a 47-60% sensitivity (3, 17). Endoscopic ultrasound sensitivity can reach 92.6%, meanwhile the most reliable method of identifying insulinomas up to date is ^{68}Ga-Exendin-4 PET/CT with an accuracy of 97.7% (18, 19).

With invasive techniques, like endoscopic ultrasound or selective intraarterial calcium stimulation with venous sampling (ASVS) the detection rate increases, although the usefulness of these examinations is highly dependent on the centers' facilities and the examiners' experience. Pre-operative localization is essential, as 9-23% of insulinomas cannot be found by intraoperative inspection and palpation (14).

The currently used ASVS procedure is based on the observation that intravenous calcium stimulates the production and release of native insulin from the hyperfunctioning pancreatic β cells, an effect not seen in case of normal β cells (3). During the test calcium stimulation is performed through the catheterized major pancreatic arteries and blood samples are collected from the right hepatic vein (20).

The advantage of the ASVS is that besides establishing the localization of the insulinoma, it complements the morphological picture with functional information, thus the sensitivity of the procedure is reported 62.5-100% with a specificity of 89.2% (14).

In our retrospective study we examined the usefulness of ASVS in patients with hyperinsulinemic hypoglycemia, where the standard imaging methods could not find the exact localization of the tumor within the pancreas.

2 Patients and methods

2.1 Patients

Nine patients treated between 2006 and 2021 at the Division of Endocrinology, University of Debrecen were retrospectively analyzed. Each patient presented the clinical symptoms

characteristic for insulinoma, and the diagnosis was supported by standard biochemical tests.

The diagnosis of the endogenous hyperinsulinemic hypoglycemia was confirmed by the detection of symptomatic hypoglycemia accompanied by documented biochemical hypoglycemia (blood glucose level below 2.5 mmol/l) and elevated insulin and C peptide levels (above 4 μU/mL and 0.2 nmol/L, respectively) during 72 hours fasting.

We included only patients in whom conventional imaging methods, like transabdominal ultrasound, CT or MRI failed to detect the source of hyperinsulinemia. Patients with proven factitious hypoglycemia caused by glucose-lowering drugs or with a history of diabetes mellitus were excluded.

2.2 Methods

The aim of the study was to evaluate the use of ASVS in case of nine patients previously diagnosed with insulinoma by clinical symptoms and confirmed by fasting test. With an aim to achieve information about the proper localization of the insulinoma we performed selective angiography with calcium stimulation. During selective angiography, after the punction of the right femoral artery, the gastroduodenal, superior mesenteric and splenic arteries were, one after the other, catheterized. Four ml of 10% calcium gluconate was administered to each artery. The sampling catheter was guided through the right femoral vein and placed into the right hepatic vein. Samples were obtained after selective stimulations of the arteries supplying the respective pancreatic regions before calcium administration, and 30, 60 and 120 seconds after injection. The highest insulin level of these set of samples was used for comparison. A more than 1.5 times increase of the baseline insulin level was considered significant and confirmed tumor localization within the pancreas. Insulin levels were measured from serum samples at the Department of Laboratory Medicine, University of Debrecen by chemiluminescence immunoassay (CLIA) on a Liaison XL analyzer (Diasorin Inc, Stillwater, MN, USA).

3 Results

In this retrospective study ASVS was performed in nine patients with endogenous hyperinsulinemic hypoglycemia. The mean patients' age was 45 ± 25 years with a 7:2 male predominance. In case of all patients, transabdominal ultrasound, CT scan and MRI failed to localize the pancreatic neuroendocrine tumor. In case of two cases (patients 1 and 2) octreotide scintigraphy was also performed and found to be negative. Since our case series encompasses a long period in which this procedure was just getting available, endoscopic ultrasound (EUS) was not performed due to lack of equipment and diagnostic expertise in our center.

We did precisely localize the insulin overproduction source in five cases comparing the insulin content of the samples obtained from the hepatic vein (Figure 1). In most of our cases the patients' serum insulin levels peaked early, as soon as 30 seconds after calcium administration with a slight decrease in the samples obtained later. These findings correlate with literature data (21).

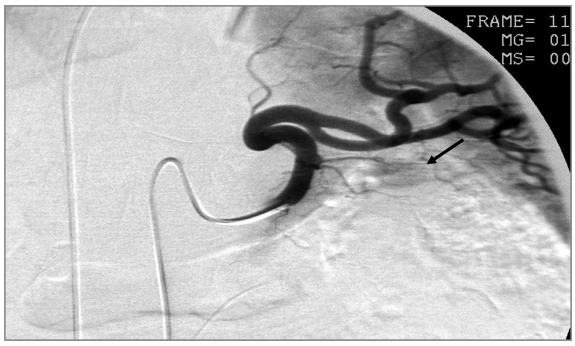

FIGURE 1
Angiography during ASVS in patient 1. After surgery, the insulinoma could be retrospectively identified on the angiography recording in the tail of the pancreas (arrow), where it was predicted by the ASVS insulin samples.

Clinical symptoms ceased and biochemical remission was achieved after surgery in 4 patients (Table 1). One patient, who had several comorbidities, has been lost due to postoperative complications, the histology of the pancreatic tumor and local metastases confirmed malignant insulinoma.

In the other four patients the insulin levels of the right hepatic vein increased to a near similar extent after calcium stimulation of each of the three arteries. This was considered compatible with hyperplasia of the pancreatic beta-cells indicating nesidioblastosis. In case of these patients, surgery was not performed except for one case; partial pancreatectomy was followed by medical treatment. Symptoms were prevented by Ca antagonist-diazoxide combination therapy (Table 1).

In all our insulinoma cases the ASVS procedure was diagnostic and helped in designing and performing the surgery and also confirmed the hormonal activity of the neuroendocrine tumors.

4 Discussion

Insulinoma is the most common functional neuroendocrine tumor of the pancreas and is the most common cause of endogenous hyperinsulinemic hypoglycemia (7). Prevention of neurological damage may be facilitated by early localization of the autonomic focus. In most cases surgical removal is curative, although the precise localization of the tumor is often challenging, as most tumors are below 2 centimeters in diameter. On the contrary, non-functioning pancreatic neuroendocrine tumors tend to be larger, therefore are more easily detectable with conventional imaging, still being recognized later due to the lack of symptoms. Insulinomas have an outstanding surgical curability with a reported 5-year disease-free survival rate of 100% due to the relatively low percentage of malignancies (3, 22).

Nesidioblastosis was first described in children and neonates, characterized by beta-cell hyperplasia and hypertrophy. The

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TABLE 1 Results of selective intraarterial calcium stimulation and patient outcome.

	Patient number								
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
age (years)	47	29	52	70	27	66	51	34	33
gender	male	male	female	male	male	male	male	male	female
Gastroduodenal artery[1]	30.9/31.2	122.5/113.1	30.4/36.1	17.5/21.7	111.1/120.4	73.1/>**500**	204.2/**304.4**	30.5/33.2	n.a.[2]
Superior mesenteric artery[1]	16.9/13.9	24.3/**102**	33.5/>**309**	16.5/17.3	131.9/118	75/>**500**	116.9/**209.7**	43.1/**105**	17.8/**75.1**
Splenic artery[1]	17.2/**174.9**	34.8/>**291**	35.9/49.8	23.1/>**291**	119.1/**231.4**	N.A.	102.2/>**500**	42.8/**68.8**	12.1/**23.1**
Histology	insulinoma	insulinoma	insulinoma	malignant insulinoma	insulinoma	n.a.	n.a.	nesidioblastosis	n.a.
Tumor size	14-16 mm in tail region	15mm in the border of tail and body	insulinoma in the head region	insulinoma in the tail region	insulinoma in the tail region	no surgery	no surgery	n.a.	no surgery
Outcome	no hypoglycemia	no hypoglycemia	no hypoglycemia	no hypoglycemia; deceased	no hypoglycemia	medical therapy	medical therapy	medical therapy	medical therapy

[1]The values for each artery are prestimulation/poststimulation hepatic vein insulin levels (μU/mL).
[2]Unsuccessful catheterization attempt due to vasospasm.
Significantly elevated post-stimulation insulin levels are bolded.

extremely rare focal cases might be treated surgically, but in the majority of the cases pharmaceutical approach needs to be implemented (23, 24). Diffuse hyperplasia of the pancreatic islet cells is often difficult to identify with routine imaging techniques, therefore in cases of nesidioblastosis ASVS is a particularly useful diagnostic tool. During ASVS insulin level elevation was found to be significantly higher in insulinomas than in nesidioblastosis (25). We found comparably marked insulin elevation in two of the nesidioblastosis cases. EUS is a relatively new technique. It is minimally invasive, can identify neoplasms smaller than 2 cm with a high sensitivity and specificity. It also allows tissue sampling for further histological evaluation. However, the sensitivity of EUS can vary from 40% to 92.6% depending on the tumor's location. Its accuracy is also highly dependent on the examiner's expertise and cannot be used to assess distant metastases (18, 26, 27). Furthermore, in the absence of hyperechogenic lesions it has also limited use in the diagnosis of nesidioblastosis (23, 28, 29).

ASVS provides information about hormonal activity of the lesion as well, and thus helps localize the tumor and might help in a more precise surgical approach with a significant decrease of reoperations (30). Another study published by Morera et al. found a 90.9% sensitivity for ASVS in localizing the tumor, which is higher than the one obtained by several studies with EUS. Moreover, its sensitivity was comparable to that of intraoperative ultrasound (IOUS) performed together with palpation (31). Although it has a 47-60% sensitivity, the locally attainable octreotide scintigraphy performed in two of our cases failed to detect and localize the insulinoma (3, 17). According to several studies involving 10-20 patients, the ASVS method's overall accuracy is around 90%. In a meta-analysis involving 339 patients a sensitivity of 93% and specificity of 86% was found (3, 32, 33). Those cases where standard imaging techniques could not detect a solitary lesion and during ASVS equally increased insulin concentrations were obtained by calcium stimulation on more than one supplying artery, were considered nesidioblastosis (24). In these patients, no tumors were found during laparotomy either. Moreover, after conservative therapy the hypoglycemic symptoms relieved, which also supports our theory.

Of course there are limitations of this technique as well. Hatoko et al. tried lower doses of calcium administration because of adverse reactions, such as nausea, hypoglycemia, hypercalcemia (34). Due to the invasive nature of the examination complication of vessel punctuation, such as bleeding or hematoma can also occur. In addition, false negative and positive results can also be found, caused by technical flaws or anatomical variants, although these are rare in case of investigations performed in skilled and well-equipped centers (35). Complications of ASVS are very rare; according to Perkov et al., complication rate is negligible, but certain precautions are needed (36). In a study of seventeen patients no complications occurred after performing ASVS, data which correlates well with our findings (37).

Novel imaging techniques using somatostatin-receptors, like octreotide-scan; ^{68}Ga-DOTATATE positron emission tomography (PET) or ^{68}Ga-DOTATOC PET are recently used for the detection and follow-up of neuroendocrine tumors. According to a study,

Exendin-4 PET/CT was superior to ^{68}Ga-DOTATATE PET/CT and ^{18}F-FDG PET/CT for the localization of the insulinoma, particularly in case of small and G2 tumors (38). Exendin-4 is a molecular tracer which targets glucagon-like peptide-1 receptor (GLP-1R), which has the highest expression on insulinoma beta islet cells and consequently has a very high sensitivity and specificity in localizing pancreatic insulinomas. Sensitivity can be as high as 97.7%, which exceeds any other imaging method. ^{68}Ga-NOTA-exendin-4 PET/CT is currently the most sensitive imaging method for preoperative localization of insulinomas with a sensitivity of 97.7%. However, the availability of these techniques for detecting insulinomas is limited (39, 40). The expression of GLP-1R is higher in nesidioblastosis than in normal pancreatic tissue, but lower than in insulinoma cases, which can be also a drawback of this imaging method (41).

Molecular imaging is an emerging and promising tool in the detection of insulinomas; however, a significant percentage of insulinomas do not express somatostatin receptors (41). GLP-1R is overexpressed in 93% of the cases, consequently GLP-1 PET/CT improves insulinoma detectability vastly; however, overexpression is only present in 36% of patients with metastases and/or malignant lesions, making the method less informative in the rare but more malevolent malignant cases (41–43). Moreover, metastatic insulinomas often lack GLP-1 receptors, and often SST2 receptor overexpression can be found (positive SRS scan in 73%) (44).

Albeit in almost every case the combination of different imaging techniques is required, in the preoperative phase the precise localization of the insulinomas is unachievable in 10-27% of the cases (14). In a systematic review of 6222 cases evaluated between 1960 and 2011, ASVS localized correctly 84.7% of insulinomas, when applied, with a mean sensitivity of 89.2% (14). ASVS might provide additional functional information in MEN-1 cases when multiple neuroendocrine neoplasms are present in the pancreas and distinction is needed between potential functioning and non-functioning tumors (3).

There are different viewpoints about the best methods to localize the tumor; the available diagnostic procedures are different in every center, and the success rate can be highly dependent on the centers' preparedness and experience (3, 22, 45). In the presented case series, our institution has served as referral center for neuroendocrine tumors, which explains the high success rate in localizing insulinomas and recognizing nesidioblastosis cases, which underscores the importance of the centralized management of rare endocrine tumors like insulinoma.

To avoid the late exocrine and endocrine pancreas function insufficiency and to facilitate postoperative healing, the best curing procedure is the pancreas saving surgical intervention, for which the exact localization of the tumor is indispensable. In the most recent European Neuroendocrine Tumor Society (ENETS) guidance paper, as first-line treatment modality for patients with preoperatively localized insulinomas a minimally invasive surgical approach is strongly suggested; laparoscopic procedures are reported to be safe and effective treatment options (7, 13, 14, 46).

The prevalence of nesidioblastosis is growing; the incidence increases after bariatric surgery (23, 47). In our institution we found a relatively high number of nesidioblastosis cases among the investigated hyperinsulinemic patients. In nesidioblastosis surgical intervention does not lead to complete healing (48). Preoperative screening is fundamental to avoid unnecessary surgery. Diazoxide reduces insulin secretion by indirect action on beta-cells and enhances glycolysis. Long-acting somatostatin analogues (SSAs) (octreotide, lanreotide and pasireotide) may prevent hypoglycemia when the insulinoma tumor cells express somatostatin receptors subtype 2 (14, 17). For metastatic insulinoma cases peptide receptor radionuclide therapy (PRRT) with ^{177}Lu-DOTATATE and everolimus can be considered in advanced, progressive insulinoma cases when hypoglycemia is refractory to SSAs (7).

In a recent study of Andreassen et al., the tumors of 80 patients showed less staining for insulin and proinsulin in malignant insulinoma cases vs. benign lesions, possibly due to a diminished insulin storage capacity; to the contrary, glucagon staining was present only in malignant tumors. Malignancy is also associated with a lack of staining for CgA and higher Ki-67 staining as a result of poor differentiation (15). These findings also underscore the usefulness and importance of the ASVS technique.

In our series of patients ASVS provided important functional information and could successfully localize the origin of the elevated insulin levels, which were usually higher in localized insulinomas, then in nesidioblastosis. No complications occurred during ASVS. Although the number of patients studied is relatively small, according to our results ASVS is still an effective tool when the source of insulin over-secretion cannot be localized with non-invasive imaging. This is in line with the most recent ENETS recommendation (7).

One limitation of our case series investigation is that several, recently available imaging methods were not, or just partially performed (EUS, IOUS, radionucleotide-labeled techniques), so true comparison of these methods cannot be estimated. The limited number of cases is also a handicap of our study.

5 Conclusions

The localization of insulinoma is fundamental, as the treatment of choice is the surgical removal of the tumor. ASVS remains a reliable tool in localization and possesses important additional functional information that are not achievable with the use of other, novel and more expensive imaging techniques.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving humans were approved by Institutional Review Board of the University of Debrecen. The studies were conducted in accordance with the local legislation and institutional

requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

SH: Writing – original draft. EB: Writing – review & editing. ÁD: Methodology, Writing – review & editing. LS: Methodology, Writing – review & editing. PF: Methodology, Writing – review & editing. EN: Conceptualization, Writing – review & editing. FG: Investigation, Writing – review & editing. ZK: Investigation, Writing – review & editing. JT: Investigation, Writing – review & editing. HB: Investigation, Writing – review & editing. MB: Conceptualization, Writing – review & editing.

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Conflict of interest

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

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EDITED BY
Antonino Belfiore,
University of Catania, Italy

REVIEWED BY
Akram Al-Ibraheem,
King Hussein Cancer Center, Jordan
Alberto Bongiovanni,
IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori", Italy

*CORRESPONDENCE
Aviral Singh
aviral.singh@genesiscare.com

†These authors have contributed equally to this work

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The PANEN nomogram: clinical decision support for patients with metastatic pancreatic neuroendocrine neoplasm referred for peptide receptor radionuclide therapy

Aviral Singh[1,2,3*†], Sebastian Sanduleanu[4†], Harshad R. Kulkarni[5], Thomas Langbein[6], Philippe Lambin[2] and Richard P. Baum[7]

[1]Theranostics (Oncology), GenesisCare Pty Ltd, Murdoch, WA, Australia, [2]Department of Precision Medicine, GROW—School for Oncology and Developmental Biology, Maastricht University, Maastricht, Netherlands, [3]Department of Nuclear Medicine, Fiona Stanley Hospital, South Metropolitan Health Service, Murdoch, WA, Australia, [4]Department of Radiology and Neuroradiology, GFO Clinics Troisdorf, Academic Hospital of the Friedrich-Wilhelms-University Bonn, Troisdorf, Germany, [5]Bold Advanced Medical Future (BAMF) Health, Grand Rapids, MI, United States, [6]Clinic for Nuclear Medicine, Zentralklinik Bad Berka, Bad Berka, Germany, [7]Advanced Theranostics Center for Radiomolecular Precision Oncology, CURANOSTICUM Wiesbaden-Frankfurt, HELIOS DKD Klinik, Wiesbaden, Germany

Introduction: Patients with pancreatic neuroendocrine neoplasms (P-NEN) may benefit from peptide receptor radionuclide therapy (PRRT). Prediction of overall survival (OS) using statistical models has the potential to guide treatment decisions. In this study, we have generated a clinicopathological and imaging parameter-based internally validated nomogram of patients who received PRRT for metastatic P-NEN to facilitate treatment decision support for the clinical management of such patients.

Patients and methods: We reviewed 447 pancreatic NEN patients treated with PRRT. Clinical variables for the prediction of overall survival (OS) included age, gender, Karnofsky performance score (KPS), weight loss, hepatomegaly, time from diagnosis to first PRRT (days), tumor functionality, presence of Hedinger syndrome, presence of liver metastases, presence of bone metastases, presence of lung metastases, alkaline phosphatase, 2-deoxy-2-[18F]fluoro-D-glucose ($[_{18}F]$FDG) positron emission tomography (PET) scan positivity, erythrocytes, platelets, creatinine clearance, leucocytes, and histologic grade of tumor differentiation based on KI-67 staining. A random survival forests (RSF) method was used to construct a model with an optimal number of clinical variables. The model was developed on 80% of the data and tested on the remaining 20% of the data. Performance of prediction was calculated using the c-index, a generalization of the area under the ROC curve (AUC) for survival models.

Results: Median follow up time was 2045 days (min 136 days, max 10329 days). Time from diagnosis to 1st PRRT, alkaline phosphatase, KPS, hepatomegaly, weight loss, $[_{18}F]$FDG-PET scan positivity, Ki-67% derived histologic grade, lung metastases, age, presence of bone metastases, platelet count, erythrocyte count, creatinine clearance, hemoglobin, presence of functioning tumor, creatinine,

and gender, were in order of importance, all independent predictors for overall survival. The development set c-index was 0.86, while the test set c-index was 0.82. A nomogram was constructed based on the optimal number of clinical parameters selected in the RSF model.

Conclusion: This study proposes an internally validated nomogram (PANEN-N) to accurately predict overall survival for P-NEN patients following PRRT, which could be used for patient counseling to facilitate informed and shared decision support in daily clinical practice as well as for generating new hypotheses.

KEYWORDS

pancreatic neuroendocrine neoplasm, clinical decision support nomogram, peptide receptor radionuclide therapy, pedictict overall survival, machine learning

Introduction

Pancreatic neuroendocrine neoplasms (P-NEN), previously also known as islet cell tumors, are a rare group of neoplasms that account for less than 3% of all pancreatic tumors (1).

The majority of P-NENs (70-90%) are non-functioning (i.e., not associated with a hormonal syndrome such as in the case of insulinomas, glucagonomas, gastrinomas, somatostatinomas, and VIPomas) (2), posing a challenge in the diagnosis of these tumors at an early stage.

While the majority of these neoplasms are sporadic, they may be associated with a number of genetic syndromes such as multiple endocrine neoplasia-1 and von Hippel-Lindau syndrome (3).

Based on the 2017 World Health Organization (WHO) classification, P-NEN are divided into well-differentiated P-NETs: grade 1 (G1), Ki-67 <3% and/or mitotic rate <2 mitoses/2 mm2; grade 2 (G2), Ki-67: 3–20% and/or mitotic rate 2–20 mitoses/2 mm2; grade 3 (G3), Ki-67 > 20%; and poorly-differentiated pancreatic neuroendocrine carcinoma (P-NEC) including small-cell type (SCNEC) and large-cell type (LCNEC), Ki-67 > 20% and/or mitotic rate >20/2 mm2 (4).

The incidence rates of P-NEN have been increasing worldwide, which is most likely caused by the increased detection of asymptomatic disease on cross-sectional imaging and endoscopy performed for other indications (5).

Novel biomarkers, such as circulating DNA, genomic and transcriptomic profiles, mRNA and circulating tumor cells, are being developed; however, still only available in the pre routine clinical setting (6, 7). Blood sampling or liquid biopsy for the assessment of neuroendocrine gene transcripts have demonstrated significant diagnostic and prognostic potential in recent studies, such as the NET-test, nevertheless these currently are not available in all countries for regular clinical application (8, 9).

The introduction of various modern imaging modalities has improved tumor localization as well as staging and restaging of neuroendocrine neoplasms. Although, Ga-68 labeled somatostatin receptor (SSTR) PET/CT has been widely used in Europe for the past two decades, the FDA only approved the use of PET/CT imaging with Ga-68 labeled DOTATATE in June 2016 (10). Gallium-68 (^{68}Ga)-edotreotide has been authorized for molecular imaging of gastroenteropancreatic neuroendocrine (GEP-NET) tumors in the European Union since December 2016 (11).

A 'NETPET' grade has been proposed as a promising prognostic imaging biomarker in NEN with PET scans using [$_{18}$F]FDGand SSTR imaging agents, which permits assessment of the glycolytic as well as somatostatin receptor status of the tumor using this dual radiotracer imaging in each patient to describe tumor heterogeneity, and thereby highlighting the more aggressive phenotype of NEN in that specific patient (12).

Historically, the management of P-NEN has been a complicated task mainly due to the heterogeneity of these tumors. The mainstay of treatment has been surgical excision of small and localized tumors. However, the majority of patients recur, even if the local resection is complete (13). Additional systemic treatments include biotherapy with somatostatin analogues, mTOR inhibitors (everolimus), multi-tyrosine kinase inhibitor (e.g., sunitinib), systemic chemotherapeutic agents such as capecitabine and temozolomide, liver metastases directed therapies e.g., chemoembolization, and receptor mediated radionuclide treatment strategies (14). Peptide receptor radionuclide therapy (PRRT) has been practiced for over two decades on a compassionate use basis in Europe as well as certain other countries (15, 16). However, the first phase-III, prospective, randomized controlled trial (NETTER-1) comparing [$_{177}$Lu]Lu$_{3+}$ labeled SSTR analog radionuclide therapy with high-dose cold SSTR-analog in gastroenteropancreatic neuroendocrine tumors (GEP-NET), demonstrated a significantly higher progression-free survival (PFS) in the [$_{177}$Lu]Lu-DOTATATE group with minimal adverse effects and excellent tolerability (17), which subsequently led to the approval of PRRT in GEP-NETs by the Food and Drug Administration (FDA) (18) and the European Medicines Agency (EMA) (19). The recently published NETTER-2 trial (20)

10.3389/fendo.2025.1514792

demonstrated that treatment with PRRT using [$_{177}$Lu]Lu-177 DOTA-TATE in the first line setting versus standard of care (control arm) significantly improved median progression-free survival (PFS) and demonstrated clinically meaningful objective response rates (ORR) in patients with higher grades (grade 2 and 3) GEP-NET. In the study, 54.4% patients had P-NEN and 29.2% patients had small-bowel NEN. Median PFS (22.8 months vs 8.5 months) and ORR (43.0% vs 9.3%) were significantly higher in patients in the treatment arm when compared to patients in the control arm, respectively. This reaffirms the therapeutic efficacy of PRRT in P-NEN even at the initial stages of therapy.

Over the past years, studies have successfully demonstrated the clinical applicability of a mathematically validated nomogram, may provide objective assessment for the surgical management of P-NEN patients (21), as well as for small intestine neuroendocrine tumor patients being considered for either surgical management or somatostatin analogue therapy (22).

With regards to the application of PRRT, there remains a lack of a similar structured and validated clinical and patient decision support system, which can be applied more universally and used widely in everyday clinical practice.

In this study, we have generated a clinicopathological as well as imaging parameter-based internally validated nomogram of patients who received PRRT for metastatic P-NENs in order to facilitate treatment decision support for the clinical management in this group of patients. This nomogram, called the PANEN Nomogram (PANEN-N), is based on the analysis of the currently largest number of P-NEN patients treated with PRRT at a single center.

Materials and methods

Patient cohort

In this single center retrospective cohort study from November 2002 to September 2019, a total of 447 patients with metastatic G1 to G3 P-NEN (M 250 (56%), F 197 (44%); age range 19–88 years, mean age 62 years), who underwent PRRT at Zentralklinik Bad Berka, Germany, were retrospectively reviewed.

Patient selection for PRRT was in accordance with the published guidelines for PRRT (23), including relevant clinical parameters such as life expectancy of more than 6 months, somatostatin receptor positive pancreatic NEN, and adequate renal function and bone marrow reserve. The diagnosis of P-NEN was confirmed based on histopathological reports performed on the tumor tissue of the respective patients. The final decision to perform PRRT was made by the multidisciplinary neuroendocrine tumor board established and regularly audited by the European Neuroendocrine Tumor Society (ENETS). The baseline demographics and clinical characteristics of the patients are listed in Table 1.

In total 447 patients received PRRT and were included in the final analysis. Multivariate analyses for overall survival were based

TABLE 1 Baseline demographics and clinical characteristics of the patients.

Variable name	Count	Percentage
Age (years (± SD))	62 (± 12)	
Gender		
Male (n)	250	56%
Female (n)	197	44%
Tumor grade (based on Ki67 proliferation index)		
G1	75	17%
G2	208	47%
G3	46	10%
Unavailable	118	26%
Tumor functional status		
Functioning tumors (n)	98	21.9%
Non-functioning tumors (n)	349	78.1%
Previous surgery		
Excision of liver metastases	262	59%
Pancreatectomy	163	36%
Small intestine resection	7	2%
Large intestine resection	15	3%
None	118	26%
Other (not tumor-specific)	121	27%
Previous systemic treatment		
Chemotherapy	131	29%
Everolimus	3	1%
Interferon	24	5%
Lanreotide/Somatuline	8	2%
Sandostatin	163	36%
Other	3	1%
None	115	26%
Karnofsky performance score		
<= 50	24	5%
60	18	4%
70	26	6%
80	79	18%
90	207	46%
100	92	21%
Unavailable	1	0%
Median survival (days, (± SD))	1011 (± 1002)	

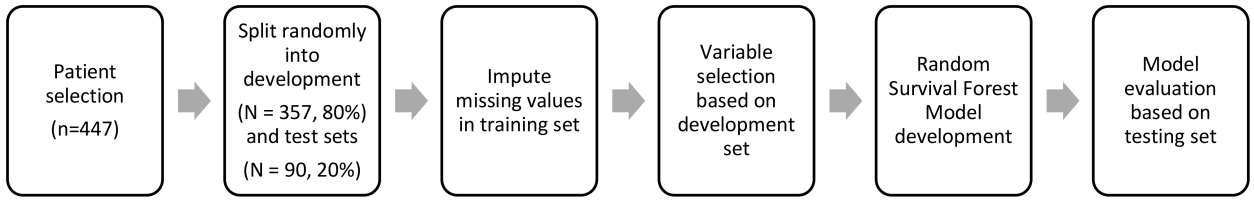

FIGURE 1
Schematic overview of the Random Survival Forest (RSF) model development process used in this study.

on Random Survival Forests (RSF), an ensemble tree method for analysis of right-censored survival data. The model was learned on a randomly selected 80% of the data and tested on the remaining 20% of data. Model results were expressed by the c-index. A schematic overview of the model development process used in this study is shown in Figure 1.

Statistical analysis

Statistical analyses were conducted in R (version 3.4.0). A two-sided p-value cut-off of 0.05 was set to determine statistical significance. The prognostic value of the individual clinical features was evaluated using concordance index (CI) with the survival package (Therneau T (2015)). A Package for Survival Analysis in R. version 2.38, URL: https://CRAN.R-project.org/package=survival) and randomForestSRC package (Ishwaran H (2017) Fast Unified Random Forests for Survival, Regression and Classification (RF-SRC) version 2.9.1, URL: https://cran.r-project.org/web/packages/randomForestSR). Nomograms were constructed with the 'nomogram' function in the 'rms' package and the 'DynNom' package which generates a dynamic nomogram application for a variety of statistical models to allow a reader to interact with the model in a user-friendly manner as a standalone application or web-based interface.

Multivariable clinical Random Survival Forest (RSF) models were generated based on selecting all clinical features with a relative feature importance >0. Variable importance was computed based on the decrease of node impurity when the covariate in question is considered for the splitting.

Random Survival Forest (RSF) strictly adheres to the prescription laid out by Breiman (2003) and requires considering the outcome (splitting criterion used in growing a tree must explicitly involve survival time and censoring information) in growing a random forest model. Further, the predicted value for a terminal node in a tree, the resulting ensemble predicted value from the forest, and the measure of prediction accuracy must all properly incorporate survival information.

After selecting the important variables in the RSF analysis, the nomogram was based on a Cox proportional hazards model with the selected variables. The reason for this is that unlike traditional parametric models (such as the Cox proportional hazards model), RSF does not provide explicit coefficients for each predictor variable. Instead, RSF generates survival trees and makes predictions based on an ensemble of these trees which makes it challenging to create a simple, interpretable nomogram that directly translates the RSF's predictions into probabilities.

Variable selection

Variables included in the analysis were age, gender, KPS, weight loss, hepatomegaly, time from diagnosis to first PRRT (days), tumor functionality, presence of Hedinger syndrome, presence of liver metastases, presence of bone metastases, presence of lung metastases, alkaline phosphatase, [$_{18}$F]FDG scan positivity, erythrocytes, platelets, creatinine clearance, leucocytes, and histologic grade of tumor differentiation based on KI-67 staining. These parameters were established at the time of decision to commence PRRT for each patient.

Results

In total n=250 male (56%) and n=197 female participants (44%) were included in this retrospective cohort analysis. Median survival time for the entire cohort was 33.2 ± 32.9 months.

Biopsy-based tumor grades were available for n=329 (74%) patients and varied between G1 (17%), G2 (47%), and G3 (17%) according to Ki-67 proliferation index. Out of the 447 patients, 98 (21.9%) had functioning tumors and remaining 349 (78.1%) had non-functioning tumors. Within this cohort, (47%) 208 patients had prior surgical intervention related to their disease, out of which more than one-third (36%) patients had undergone pancreatectomy. With regards to other treatments, n=171 (38%) patients had received prior treatment with long-acting somatostatin analogues, particularly, Octreotide (Sandostatin® LAR®) and Lanreotide (Somatuline® AG®) in n=163 (36%) and n=8 (2%) patients, respectively. Previous chemotherapy was administered in n=131 (31%) patients. Interestingly, the mTOR inhibitor, Everolimus had been the therapy of choice for a meager 1% patients, which could probably relate to the approval of Everolimus for the treatment of unresectable or metastatic, well- or moderately-differentiated neuroendocrine tumors (NET) of pancreatic origin in adults with progressive disease following the results of the RADIANT-3 trial from 2011 (24) and probably also due to the comparatively more severe toxicity profile. Other therapeutic options such as interferon-alpha were used in lesser number of patients. The baseline demographics and clinical characteristics of the patients are reported in Table 1.

Median follow up time was 2045 days (min 136 days, max 10329 days). In total 308 patients died by the end of follow up. Three-hundred and fifty-seven randomly selected patients (80%) were included in the development set, while 90 patients were held out for the test set (20%).

In total n=286 patients had undergone an [$_{18}$F]-FDG-PET study.

In total 17 variables were selected based on basis of their relevant importance in the RSF analysis. The development set c-index was 0.86, while the test set c-index was 0.82.

Figure 2A depicts a web-based nomogram with these 17 selected relevant variables which is accessible through the URL: https://dynamicnomogramnet.shinyapps.io/dynnomapp/.

This is a simple-to-use web-based nomogram for convenient application, which can aid personalized treatment and clinical decision-making.

Values for the 17 prognostic variables can be chosen via horizontal sliders, which in turn computes individualized linear predictors from a Cox proportional-hazards model, and dynamically renders (1) a Kaplan-Meier survival curve (2) numerical summaries, and (3) model parameter summaries in real time.

Another option is provided to predict overall survival at specific follow-up times, which subsequently can be viewed in the "Numerical Summary" tab.

Figure 2B depicts a predictive nomogram with these 17 variables constructed for manually calculating 2- and 5-year overall survival probabilities.

Time from diagnosis to first PRRT, alkaline phosphatase, KPS, presence of hepatomegaly, weight loss (unintentional loss of ≥2 kg weight in past 3 months), [$_{18}$F]FDG-PET positivity (at variable timepoints), tumor grade based on proliferation index (Ki-67), presence of pulmonary metastases, age at PRRT, presence of bone metastases, platelet count, erythrocyte count, creatinine clearance, hemoglobin, functioning (functional) tumor, plasma creatinine, gender, presence of myocardial metastases, presence of liver metastases, presence of Hedinger syndrome (carcinoid heart disease), leucocyte count, and Modification of Diet in Renal Disease trial (MDRD)-based estimated glomerular filtration rate (eGFR) were according to their importance in the Random Survival Forest model all independent predictors for overall survival (Figure 3).

Discussion

Pancreatic neuroendocrine tumors are a group of rare and heterogenous tumors with a poorly defined natural history and unclear biological behavior (25, 26).

Nowadays, P-NEN are being detected with increasing frequency and new treatment regimens including PRRT are being established. However, currently there is no set method to determine the prognosis of patients using the variable possible prognosticating parameters in the pre-PRRT setting.

In this study we have designed a single center, internally validated nomogram (PANEN-N) based on clinicopathological as well as imaging parameters for the prediction of overall survival (OS). In the RSF model, Time from diagnosis to first PRRT, alkaline phosphatase, KPS, hepatomegaly, weight loss, [$_{18}$F]FDG-PET scan positivity, histologic grade, presence of lung metastases, age, presence of bone skeletal metastases, erythrocytes, platelets, creatinine clearance, hemoglobin, gender, functioning (functional) tumor, and creatinine were in order of importance all independent predictors for overall survival. This model had a high discriminative performance (AUC = 0.82) in the testing cohort.

Although several studies not specifically addressing PRRT or medical treatment have reported prognostic factors in the management of P-NENs (27), to our knowledge there is currently not a single nomogram in the literature identifying clinicopathological and imaging markers for clinical decision support in patients with metastatic P-NEN treated with PRRT in a meaningfully large cohort of patients. Furthermore, this study includes the largest number of pancreatic neuroendocrine neoplasm patients treated with PRRT at any single center and studied for prognostication of survival following PRRT with the aim of designing a predictive clinical support decision tool that could be used to include in the algorithm of informed consent by the patient.

The best way to sequence systemic therapeutic options in patients with P-NEN has not yet been fully established. For patients with unresectable disease, options to control tumor growth and symptoms related to tumor bulk or hormonal hypersecretion include somatostatin analogs, nonsurgical liver-directed therapy, and systemic antitumor therapy using everolimus or sunitinib, cytotoxic chemotherapy, or peptide receptor radionuclide therapy (PRRT).

A European phase III trial, SEQTOR (NCT02246127), is comparing the efficacy and safety of chemotherapy (fluorouracil and streptozotocin) followed by everolimus versus everolimus followed by fluorouracil and streptozotocin in patients with advanced and progressive pancreatic NET (28), and there is also an ongoing trial of PRRT versus sunitinib for progressive disease (29).

PRRT using radiolabeled somatostatin analogs is an option for patients with disease that expresses somatostatin receptors and has progressed on other treatment modalities including at least one somatostatin analog (30). Although clinical trials are being planned, there are no data yet specifically comparing PRRT with other therapeutic agents, and the choice of therapy in this situation has been previously based on the availability of PRRT and patient preference.

As evident from the nomogram, time to treatment is an important factor for these patients and reflects the apparent delay in the decision to perform PRRT since diagnosis. Time to treatment with PRRT could have been prolonged in several cases, since sequencing of earlier PRRT is not yet supported due to the lack of evidence based on phase-3, prospective, randomized clinical trials. Alkaline phosphatase (AP) is a known independent non-specific tumor marker and AP levels above normal have been reported as predictive of shorter survival in both univariate and multivariate analysis in patients with metastatic neuroendocrine tumors (31). Adriantsoa et al. reported on the prognostic value of AP in G1 and G2 NET patients, including 29 patients with

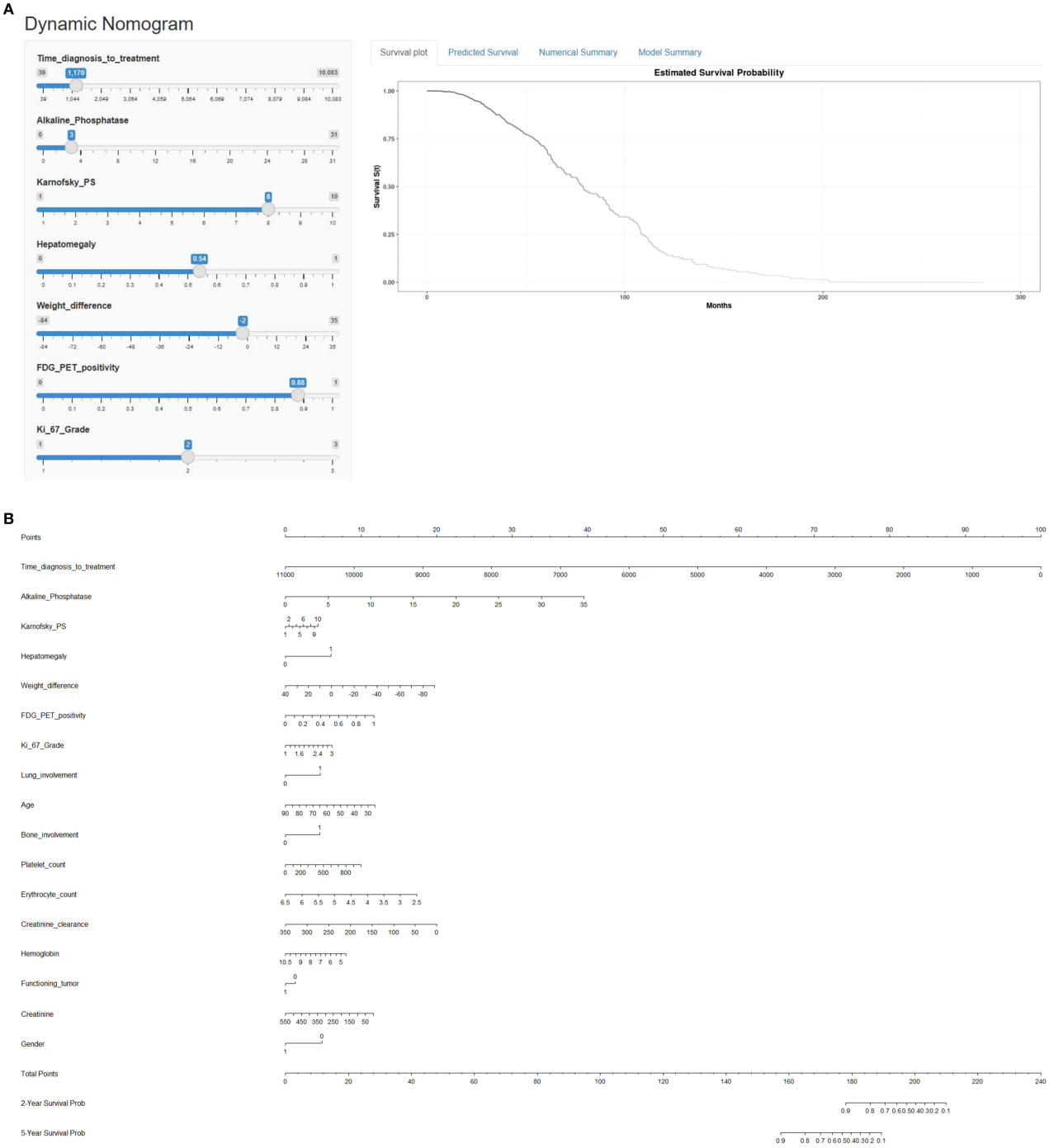

FIGURE 2
(A) Web-based survival rate calculator (Dynamic Nomogram (shinyapps.io)) to predict the overall survival of metastatic P-NEN patients treated with PRRT. Time_diagnosis_to_treatment refers to time from diagnosis to first PRRT treatment (in days). Alkaline phosphatase (ALP) values are shown in µkat/L, weight difference in kg, platelet count in G/L, erythrocyte count in T/L, creatinine clearance in mL/min/1.73 m^2 and creatinine in µmol/L. **(B)** Nomogram for prediction of overall survival (OS) in metastatic pancreatic NEN treated with PRRT. The nomogram is based on a cox proportional hazards model and is used by drawing a vertical line from each predictor value to the score scale at the 'top-points'. After manually summing up the individual scores, the 'total points' correspond to the probability (prob) of overall survival, which are estimated by drawing a vertical line from this value to the bottom scale '2-year survival prob' or '5-year survival prob' to estimate overall survival.

duodenal/pancreatic NET, and reported that in multiparametric analysis progression-free survival correlated with serum AP level ($p = 0.017$) (32), thus emphasizing its significance as an independent prognostic marker. Furthermore, an elevated serum AP reflects the possibility of skeletal metastatic disease as well as the possibility of coexisting hepatic metastases. Skeletal metastases are not always easily appreciable on staging and restaging CT scans. Therefore, the value and trends in progression or regression of the alkaline

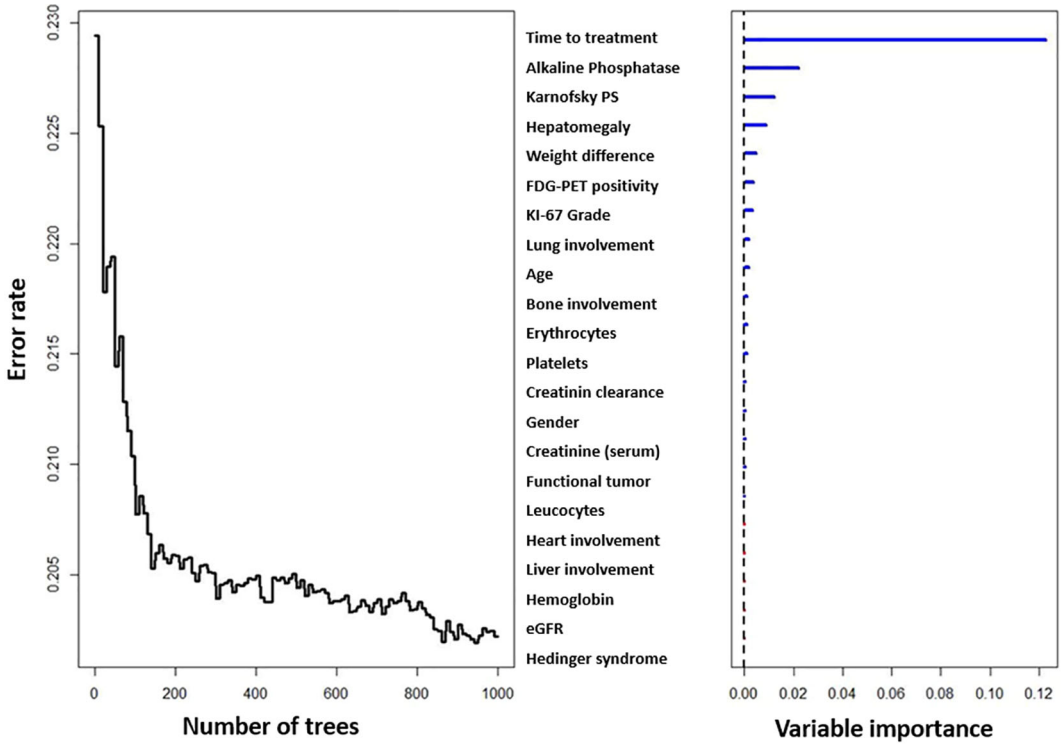

FIGURE 3
Variable importance and model error rate with increase in number of trees.

phosphate should be monitored closely in comparison to SSTR-based PET/CT imaging and considered as a prognostic biomarker for patients with P-NEN.

KPS is a validated performance index of the physical ability of a patient and in a multivariate analysis Ezzidin et al. reported that KPS of less than or equal to 70 was an independent predictor of poor survival of GEP-NET patients treated with PRRT using [$_{177}$Lu] Lu-DOTATATE (33). Weight loss of >2kg in past 3 months prior to the decision of commencement of PRRT was a marker of poor survival and this is also in line with previous studies (34) that reported baseline weight loss as a significant predictor of disease-specific survival in various GEP NET's prior to radionuclide therapy with the radiolabeled somatostatin analog[$_{177}$Lu]Lu-DOTATATE.

The presence of hypermetabolic tumor burden on an [$_{18}$F]FDG PET/CT in histologically proven well-differentiated low-grade NEN represents tumor heterogeneity and is associated with either the pre-existence of aggressive tumor burden or dedifferentiation of disease during its course. In a prospective 10-year follow-up study in 166 patients with histologically proven gastroenteropancreatic neuroendocrine neoplasms (GEP-NEN) including 28 pancreatic NET patents ([$_{18}$F]FDG negative n=7, a; [$_{18}$F]FDG positive n=21), Binderup et al. demonstrated that a positive [$_{18}$F]FDG PET scan was associated with a shorter OS than a negative [$_{18}$F]FDG PET scan (hazard ratio: 3.8; 95% CI: 2.4– 5.9; P, 0.001). In G1 and G2 patients (n 5 140), a positive [$_{18}$F]FDG PET scan was the only identifier of high risk for death (hazard ratio: 3.6; 95% CI, 2.2–5.9; P, 0.001). In this study, PRRT was performed in 78 (47% of enrolled) patients, and it was observed that in addition to a longer survival for the patients receiving PRRT, the survival benefit seemed most pronounced in the [$_{18}$F]FDG –positive patients in whom the median survival time for those who received PRRT was 4.4 y compared with 1.4 y for patients not receiving PRRT (35). One of the first study to investigate the prognostic value of an integrated parameter derived from dual somatostatin receptor imaging and [$_{18}$F]FDG PET in 24 (39%) pancreatic NET patients from a cohort of GEP-NET patients was performed by Chan et al, who developed the NETPET score, concluded that NETPET score was a significant predictor of overall survival on both univariate and multivariate analyses, emphasizing on the prognostic value of FDG positive tumor status of NET (12), findings which were later validated in a multicenter study (36).

Tumor proliferation index represented as Ki-67% defines the grade of tumor and is the basis for classification of neuroendocrine neoplasms. Several studies including patients with GEP-NEN receiving PRRT for G1 and G2 NET have not demonstrated any statistically significant prolongation in median overall survival with PRRT versus high-dose long-acting octreotide, a finding which has been reflected by the results of the NETTER-1 trial (37). This is most likely considered to be a result of the crossover of patients from standard-of-care arm to investigational therapy product arm on progression of disease over the prolonged follow up trial period. The well-differentiated lower grade (G1 and G2) of NEN (38) also represents the main group in which PRRT is usually recommended. In the largest cohort of intention to treat analysis of PRRT of NEN

including n=1048 patients with WHO grades G1, G2, and G3 NEN, those with a lower Ki-67 index had a prolonged overall survival compared to higher grade neoplasms (15).

By the time the diagnosis of a NEN is established, most patients already have metastatic spread of disease, and out of all NEN, 40-50% of pancreatic NEN patients present with distant metastases at initial diagnosis (39). In general, NEN with distant metastases are considered incurable leading to a relatively shorter survival despite the currently available management options. In our study cohort, involvement of the lungs (pulmonary metastases) and bone metastases were found to be significant predictive factors for application toward the developed nomogram for OS.

In our study, younger age at diagnosis of pancreatic NEN was a predictor of improved overall survival status following PRRT. A recently published study explored the trends in the incidence and incidence-based mortality of early-onset GEP-NENs obtained from the Surveillance, Epidemiology, and End Results (SEER) database, and reported that the prognosis of early-onset GEP-NENs was significantly superior to that of later-onset GEP-NENs, regardless of the tumor site (40), where incidence-based mortality was analyzed for >1000 patients early-onset patients compared to >5400 later-onset patients (40). Although not directly related to post-PRRT survival assessment of pancreatic NEN patients, the results of the study provide an insight into the post-therapeutic survival trends in this patient population.

The current understanding in the development of PRRT related haematotoxicity, particularly myelodysplastic syndromes leading to poor survival prognosis is reported at <3% due to the relatively low estimated bone marrow absorbed radiation dose. In the analysis of long-term tolerability of PRRT in patients with neuroendocrine tumors, Bodei et al. (41) reported that risk factors associated with bone marrow toxicity were previous chemotherapy, other previous myelotoxic therapies and pre-existing anemia. When analyzing the codependent clinical variables, platelet toxicity grade was found to be a significantly associated factor with longer PRRT duration (41). In our study, of all hematological factors associated with overall survival post-PRRT, low platelet counts at diagnosis was of highest relevance followed by erythrocytopenia, and subsequently anemia. Here, it is important to note that hemoglobin and erythrocyte counts can be managed and sustained either with hemopoietic therapies such as erythropoietin and packed red blood cell transfusions, and low total and differential leukocyte counts can usually be managed with granulocyte colony stimulating factors. However, it is extremely challenging to manage significant or critical thrombocytopenia in clinical practice.

Renal irradiation arises from the proximal tubular reabsorption of the radiopeptide and the resulting retention in the interstitium. Due to their marked radiosensitivity to the range of doses resulting from PRRT, the kidneys represent the critical organs (42). Over prolonged time period, irrespective of nephroprotection, PRRT has the potential to affect the renal function with a median loss of creatinine clearance of up to 4% and 7% per year for 177Lu-octreotate and 90Y-octreotide, respectively. Risk factors promoting the decline of renal function after PRRT have been considered to be the cumulative/per-cycle renal absorbed dose, advanced age, hypertension, and diabetes (43). In our study, we observed that calculated plasma creatinine clearance value was comparatively more significant in the prognosis of overall survival post-PRRT compared to laboratory based estimated GFR.

Functional neuroendocrine neoplasms represent the group of patients that actively produce various hormones depending on the origin of the NEN and often associated with varying clinical symptoms depending on the metastatic spread of disease. It has been reported that functioning serotonin-producing P-NEN are aggressive neoplasms with a survival rate similar to that of other aggressive functioning neuroendocrine pancreatic neoplasms like ACTH-secreting P-NENs associated with Cushing's syndrome (44).

Since most NETs are not functional (often not causing signs or symptoms), early diagnosis is difficult, and theoretically may reduce survival by reducing the chance of curative treatment. In this cohort with an individualized treatment regimen a slight survival benefit for non-functioning tumors ("1") in comparison to functioning tumors ("0") was found when all other variables were fixed. Indeed, our nomogram finds (when all other variables are fixed) a slight survival benefit for non-functioning tumors in comparison to non-functioning tumors.

Regarding patient gender, patient cohort treated with PRRT with pancreatic NEN in our study demonstrated a shorter overall survival for "1 (female)" compared to "0 (male), and this finding was inconsistent with the SEER database analysis, where compared to females, males had a better overall survival prognosis for tumors originating in the colon, small intestine, pancreas, and stomach (45).

Nomogram model reporting was done according to TRIPOD (Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis)-standards (46). The TRIPOD offers a standard way for reporting the results of prediction modeling studies and thus aiding their critical appraisal, interpretation, and uptake by potential users. With the help of the TRIPOD model reporting, further correlative studies may be performed to cross-validate the results of this study with other centers, which would provide further statistical strength to these findings in different patient cohorts.

Our nomogram has, in our opinion, the potential to further aid clinical decision making for treatment with PRRT in patients with metastatic pancreatic NEN. It will provide P- NEN patients the opportunity to discuss their individual clinical situation based on the parameters analyzed and reported in the nomogram and empower them to make an informed decision for the management of their clinical condition with better understanding and greater confidence. For a wider clinical applicability and clinically practical benefit to patients and physicians, we plan to collaborate with other institutions, where a reasonable number of patients have been treated with PRRT, to collaborate with our dataset and cross-validate these findings.

Several limitations of this study were (I) The nomogram was not validated on an external dataset; (II) Our study includes patients from a single center, which is a limitation when considering the outcomes of this study. Perhaps, it would be interesting to look at PFS in future studies as it occurs earlier in the patient journey and may present the possibility of alternative therapeutic options, for

example, it may indicate the use of localized therapies such as in case of liver or bone predominant progression, etc. However, focusing on the aim of our study, we would defer this to future sub-analyses. Finally (III), we did not identify differences in subgroups receiving different radiopharmaceuticals for PRRT (for e.g., [$_{177}$Lu]Lu-DOTATOC vs[$_{90}$Y]Y-DOTATATE).

Although progression free survival (PFS) is an often reported clinically relevant parameter, its relevance is usually limited to the early phase of survival analysis. However, the patients are always more interested in the more medically relevant survival outcome parameters, namely, overall survival (OS), which therefore has greater relevance for analysis and reporting. However, in this cohort, the OS plays a rather important role, as it is a more definitive parameter from patients' perspective. Moreover, PFS in this group of patients may be misleading, since most patients were treated with PRRT as a last line therapy option, and PFS when measured via RECIST 1.1, which is primarily anatomical imaging based and unable to reliably measure bone disease, and therefore, has been debatable due to its obvious limitations in assessing targeted receptor based molecular imaging and therapy such as PRRT. Furthermore, there are currently no standardized, globally accepted protocols for response to assessment using PET/CT-based receptor-targeted molecular imaging. Moreover, despite certain variably sensitive tumor markers, there are no definitive, highly specific tumor markers for the evaluation of response to therapy or prognosis in patients with neuroendocrine neoplasms undergoing PRRT.

Prospects of this initial study include the validation of the current nomogram on one or several prospective cohorts, the addition of radiomics and deep learning imaging biomarkers to the current nomogram to better identify high survival P-NET groups with PRRT, and the generation of nomogram based on other clinically relevant outcomes such as PFS.

Conclusion

This study proposes an internally validated nomogram to accurately predict overall survival for patients suffering from metastatic pancreatic neuroendocrine neoplasm based on the clinicopathological as well as medical imaging parameters, namely PANEN-N. The model could be used to facilitate decision support in daily clinical practice and can be used for patient counseling and shared decision making for patients presenting for peptide receptor radionuclide therapy as well as for generating new hypotheses. External multicenter validation of this nomogram is mandated prior to its routine clinical application.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

In 2007, the responsible ethics committee permitted retrospective and prospective data collection and analysis by the national German neuroendocrine tumor registry (NET-Register) at our NET Center, which was renewed in 2014. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AS: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. HK: Conceptualization, Project administration, Supervision, Writing – original draft, Writing – review & editing. TL: Conceptualization, Data curation, Investigation, Resources, Writing – original draft, Writing – review & editing. PL: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. RB: Conceptualization, Funding acquisition, Project administration, Resources, Software, Writing – original draft, Writing – review & editing.

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Conflict of interest

RB is an advisor to IPSEN, ITM, and AAA, and has received honoraria and research/travel support from Ipsen, AAA and ITM. AS has received honoraria and/or travel support from ITM, HT Medica, Telix Pharmaceuticals, and GenesisCare, and holds minority shares in GenesisCare Pty Ltd. PL owns minority shares in the company OncoRadiomics.

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

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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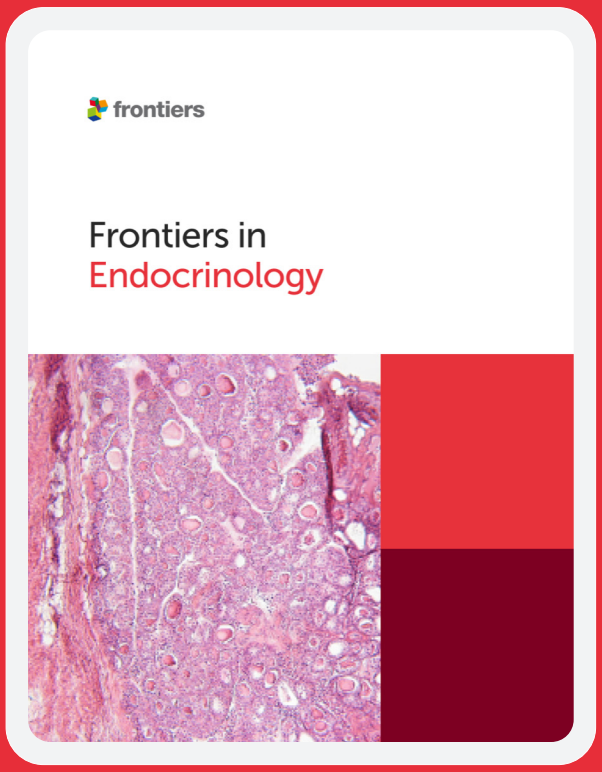

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