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Angiogenic biomarkers of response to treatment with peptide receptor radionuclide therapy in neuroendocrine tumours

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Abstract

Introduction: Neuroendocrine tumours (NETs) are a heterogeneous group of tumours, which is characterised by rich vascularisation. The role of angiogenesis in NETs has been widely researched. Peptide receptor radionuclide therapy (PRRT) is an effective treatment method for patients with disease progression in NETs. Due to the heterogeneity of NETs, the response to treatment varies. Currently, the finding of efficient markers helpful in assessing the response to treatment in NETs is crucial. The aim of this study was to assess chromogranin A (CgA) and angiogenic factors in gastro-entero-pancreatic (GEP) and broncho-pulmonary (BP) NET patients treated with PRRT.

Material and methods: The study group included 40 patients with GEP NETs and BP NETs, who completed 4 cycles of PRRT. Serum levels of CgA and angiogenic factors such as vascular endothelial growth factor (VEGF) and its receptors (VEGF-R1, VEGF-R2, VEGF-R3) were assessed before and after 4 cycles of PRRT. All tests were determined using ELISAs.

Results: The concentration of CgA, VEGF-R1, and VEGF-R2 decreased significantly, whereas VEGF-R3 increased significantly after PRRT. PRRT did not affect VEGF — it was similar before and after the radioisotope treatment. Based on AUROC, only VEGF-R1 exhibited good performance in distinguishing between NET patients before and after PRRT; the area under the curve (AUC) was 0.7.

Conclusions: VEGF-R1 is a potential biomarker for assessment of the effectiveness of PRRT in NET patients. **(Endokrynol Pol 2024; 75 (4): 412–418)**

Key words: *neuroendocrine tumour (NET); peptide receptor radionuclide therapy (PRRT); radioligand therapy (RLT); angiogenic markers; vascular endothelial growth factor (VEGF); vascular endothelial growth factor receptor (VEGF-R)*

Introduction

Neuroendocrine tumours (NETs) are rare, heterogeneous tumours originating from the diffuse endocrine system (DES). Most NETs (70%) are located in the gastrointestinal tract (gastroenteropancreatic neuroendocrine neoplasms; GEP-NETs) [1–3], and secondly (20%) in the respiratory system (bronchopulmonary neuroendocrine tumours; BP-NETs) [4]. Over 12 years of observation it was shown that the incidence of NETs increased ~2-fold [5]. NETs are richly vascularised; hence, the importance of angiogenesis has been widely studied in these tumours [6–8]. Vascular endothelial growth factor (VEGF) is the main factor associated with the angiogenesis process, and it binds to the receptors VEGFR-1, VEGFR-2, and VEGFR-3 [9, 10]. VEGF (also known as VEGF-A) is part of a family of growth factors that also includes VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PLGF) [11]. Studies have proven that both VEGF and VEGF-R may play an important role in disease progression [12, 13]. According to the literature, VEGF acts as a factor in increasing vascular permeability and as a mitogen specific for endothelial cells, and therefore it might initiate angiogenesis in malignant tumours [11]. The VEGF pathway is responsible for maintaining cancer cell autonomy primarily through autocrine signalling [14]. Angiogenesis plays a key role in the process of tumour initiation, which facilitates the spread of cancer cells, including through the so-called vascular co-option, in which existing vessels are "hijacked", or by building endothelium-like blood channels by tumour cells (vasogenic mimicry) [15]. Although the primary treatment method for NETs is surgery, other forms of systemic therapy are also used [1, 2, 16]. Among other things, increased expression of somatostatin receptors in somatostatin receptor scintigraphy (SRI) allows pa-

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tients to be qualified for targeted therapy with isotopically labelled somatostatin analogues (PRRT — peptide receptor radionuclide therapy) [17]. PRRT is most often the second line of treatment in the disease progression in patients with advanced, unresectable G1/G2 NETs showing overexpression of somatostatin receptors [2, 17, 18]. The effectiveness of this therapy has been confirmed in numerous studies, where a significant reduction in the risk of disease progression was confirmed [19]. PRRT may also be a method of neoadjuvant treatment, enabling surgical intervention in initially inoperable NETs [20]. PRRT acts through somatostatin receptors in the tumour (SST-2), the expression of which also varies significantly and thus induces DNA damage in cells, which affects the different therapeutic responses [21]. There are reports that many mechanisms related to the tumour microenvironment, such as hypoxia, the composition of the extracellular matrix, or the presence of tumour-associated fibroblasts, may affect the final effect of treatment, including being responsible for resistance to PRRT therapy [22]. The biochemical diagnosis of NETs includes the determination of non-specific markers, such as chromogranin A (CgA), belonging to the granin family, i.e. acidic glycoproteins [23]. CgA is currently the most used marker for monitoring patients with NETs, also treated PRRT, but it is still not a perfect biomarker for assessing the response to treatment [1, 23–25]. According to our current knowledge, there are no data on the assessment of angiogenic factors as potential biomarkers useful in monitoring patients treated with PRRT. The aim of the study was to assess the concentration of CgA and angiogenesis factors (VEGF, VEGFR-1, VEGFR-2, VEGFR-3) in a group of patients with NETs before and after PRRT treatment, and to assess whether angiogenic factors could be useful in assessing the effectiveness of PRRT.

Material and methods

Patients

The study included patients under the care of the Department of Endocrinology and Neuroendocrine Tumours, the European Neuroendocrine Tumour Society (ENETS) Centre of Excellence in Katowice, with histopathologically confirmed advanced neuroendocrine tumours (NET G1/G2 with Ki-67 < or = 20%) in IV clinical stage according to the Tumor–Nodes–Metastases (TNM) American Joint Committee on Cancer(AJCC)/Union for International Cancer Control (UICC) classification and those qualified for radioisotope therapy using yttrium-90 (^{90}Y)/lutetium-177 (^{177}Lu)-DOTA-0-Tyr3-Octreotate (DOTATATE).

The radioisotope treatment was applied in standard 4-course protocols. Patients received lutetium (7.4 GBq of [177Lu]Lu-DOTA-TATE) (LutaPol®, Polatom, Otwock, Poland) or tandem therapy (1.85 GBq [90Y]Y-DOTA-TATE + 1.85 GBq [177Lu]Lu-DOTA-TATE) (ItraPol®, Polatom, Otwock, Poland and LutaPol®, Polatom, Otwock, Poland). During 8–12-week intervals, long-lasting somatostatin analogues were also administered: octreotide (30 mg) or lanreotide (120 mg) every 4 weeks.

Laboratory parameters were assessed before PRRT treatment (3–6 months) and after PRRT treatment (2–6 months). The stage of the disease and the differentiation of the tumour were assessed based on the current TNM staging and grading system for NET classifications according to the World Health Organisation (WHO) 2019 criteria [2]. Disease status was assessed according to radiological Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 criteria. The exclusion criteria from the study were the presence or suspicion of another malignant tumour, advanced heart failure, and renal failure in stages IV and V. The study was conducted in accordance with ethical standards and approved by the Bioethics Committee of the Silesia Medical University in Katowice.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of VEGF, VEGFR-1, and VEGFR-2 were determined using the Quantikine Human Immunoassay (R&D Systems) and VEGFR-3 by Platinum ELISA (Bioscience), according to the manufacturer's protocol. Blood specimens were collected during hospitalisations. Fasting blood samples at 8.00 a.m. from an arm vein were gathered. Until the analysis, the serum was stored at –80°C. The serum levels of Cg were determined using a uQuant (Bio-Tek).

Statistical analyses

Statistical analyses were carried out using STATISTICA version 13.36.0 (StatSoft) software. The distribution of the data was determined by the Kolmogorov-Smirnov test. Data are presented as median and interquartile ranges for nonparametric data. The comparison of CgA, VEGF, VEGF-R1, VEGF-R2, and VEGF-R3 concentrations between the NET patients before and after PRRT (naïve and receiving PRRT) was performed using the Wilcoxon test for paired samples. To investigate the prognostic value of CgA, VEGF, VEGF-R1, VEGF-R2, and VEGF-R3 in predicting PRRT response in NET patients, receiver operating characteristic (ROC) curves were plotted, and the sensitivity, specificity, and area under the curve (AUC) were calculated. For correlation analysis, p values and correlation coefficients (r) were calculated using Spearman's correlation test. Results were considered significant at $p < 0.05$.

Results

Clinical characteristics of the study group

The demographic, biochemical, and clinical characteristics of the participants recruited for the study are presented in Table 1. The NET patient cohort consisted of 47.5% males and 52.5% females, with a median age of 54 years. All patients were diagnosed with well-differentiated NETs; 45% each of NET G1 and NET G2. All of them had advanced disease IV stages of TNM (100% of NET patients had distant metastases) at the time of PRRT starting. The most common primary site location was the pancreas (37.5%). Of these patients 42.5% had carcinoid syndrome.

CgA and angiogenic factors: VEGF, VEGF-R1, VEGF-R2, and VEGF-R3

In the next step, we used the Wilcoxon signed rank test to test 2 dependent samples (before and after PRRT), and thus we analysed whether there was a significant difference between the levels of these biomarkers (CgA, VEGF, VEGF-R1, VEGF-R2, and VEGF-R3). The Wilcoxon test showed that these differences were

Table 1. *Clinical characteristics of the neuroendocrine tumour (NET) patients/the study group*

Value	Study group (n = 40)
Age [years]	
Mean [range]	54 (25–71)
Sex	
Male	19 (47.5%)
Female	21 (52.5%)
Localization	
GEP-NET	
Pancreas	15 (37.5%)
Small bowel	13 (32.5%)
Rectum	1 (2.5%)
Unknown primary site	7 (17.5%)
BP-NET	4 (10%)
Tumour grade	
GEP-NET	36 (90%)
G1	18 (45%)
G2	18 (45%)
BP-NET	4 (10%)
Typical	1 (2.5%)
Atypical	3 (7.5%)
Stage	
IV	40 (100%)
Carcinoid syndrome	
Yes	17 (42.5%)
No	23 (57.5%)
Kind of treatment	
SSA	
Yes	40 (100%)
No	0 (0%)
Previous surgery	
Yes	19 (47.5)
No	21 (52.5)
PRRT	
Yes	40 (100%)
No	0 (0%)
Disease stage after PRRT*	
SD	20 (50%)
PD	6 (15%)
PR	14 (35%)

Data are shown as mean, number, and percentage (%).
GEP-NET — gastroenteropancreatic neuroendocrine tumour; BP-NET — bronchopulmonary neuroendocrine tumour; SSA — somatostatin analogue; PRRT — peptide receptor radionuclide therapy; SD — stable disease; PD — progressive disease; PR — partial response; *according to Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 criteria

Table 2. *The Wilcoxon matched pairs test of chromogranin A (CgA), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 1 (VEGF-R1), vascular endothelial growth factor receptor 2 (VEGF-R2), and vascular endothelial growth factor receptor 3 (VEGF-R3) for patients with neuroendocrine tumours (NET) treated with peptide receptor radionuclide therapy (PRRT)*

Matched pairs of variables	z	p
CgA before and CgA after	2.25	0.02
VEGF before and VEGF after	0.58	0.56
VEGF R1 before and VEGF R1 after	3.14	< 0.01
VEGF R2 before and VEGF R2 after	2.54	0.01
VEGF R3 before and VEGF R3 after	2.16	0.03

Z — value of the Wilcoxon test for groups with n > 25; p — significance level for the Wilcoxon test. Only for VEGF-R1 area under the curve (AUC) was a consequence

statistically significant ($p < 0.05$), comparing CgA, VEGF, VEGF-R1, VEGF-R2, and VEGF-R3 in NET patients before and after PRRT, in all biomarkers, except VEGF levels. As shown in Table 2.

Stratifying tumour marker levels by the differences before and after PRRT identified that individuals after PRRT exhibited significantly lower levels of CgA (1390.03 ± 3248.09 (233.94 [68.82–1200.25])), VEGF-R1 (78.03 ± 29.69 (72.40 [60.70–88.00])), and VEGF-R2 (6635.36 ± 1714.11 (6284.00 [5298.00–7601.00])) than those before PRRT CgA (2151.11 ± 3811.02 (429.97 [79.41–2533.33])), VEGF-R1 (96.62 ± 36.55 (90.30 [71.45–100.50]), and VEGF-R2 (7537.05 ± 1884.36 (7277.00 [6032.00–8849.00]), respectively) ($p < 0.05$) (Tab. S1 in supplementary material).

Levels of VEGF did not differ significantly between NET patients before PRRT versus NET patients after PRRT ($p > 0.05$) (Tab. 2 and Tab. S1 in Supplementary Material).

Given the concentration of VEGF-R3, in the group of patients before PRRT, VEGF-R3 concentrations were significantly lower than those after treatment (Tab. S1 in Supplementary Materials).

In the third step, the ROC analysis and AUC were used to assess the capacity of these biomarkers to predict PRRT response based on biomarker level changes. AUC analyses could differentiate PRRT-non-treated from PRRT-treated NET patients for VEGF-R1, VEGF-R2, and VEGF-R3. The authors differentiated the AUC value only between pre- and post-PRRT treatment.

Based on AUROC analysis (Tab. S2 in Supplementary Material), we noted that the highest statistically significant AUROC for differentiating NET patients before PRRT from NET patients after PRRT had VEGF-R1 (0.70) ($p < 0.01$), and their accuracy for differentiating these patient groups was 70%. Also, VEGF-R2 and VEGF-R3 could differentiate both NET patient groups. Although significant ($p < 0.05$), it should be noted that their AUCs < 0.7 indicate that they are

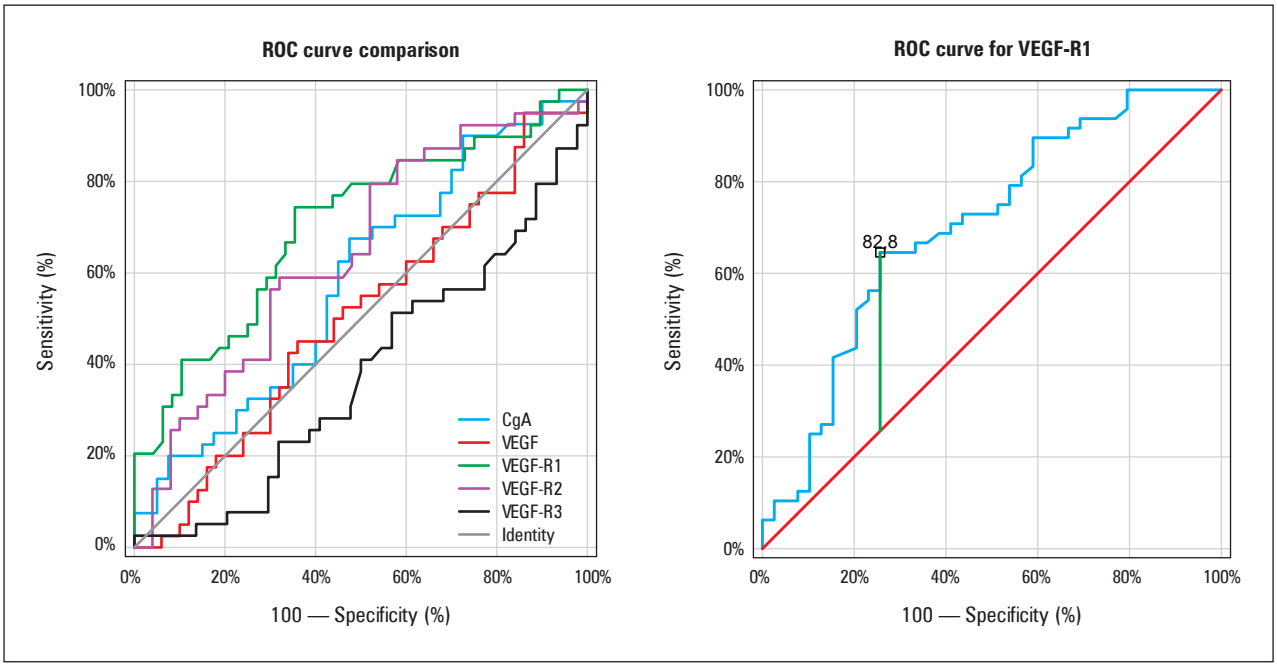

Figure 1. *Receiver operating characteristic (ROC) curves for differentiating PRRT-non-treated from PRRT-treated patients with neuroendocrine tumours (NET) (PRRT — peptide receptor radionuclide therapy);* **A.** *The area under the receiver operating characteristic (AUROC) for all markers in NET patients before PRRT and after PRRT;* **B.** *The AUROC for vascular endothelial growth factor receptor 1 (VEGF-R1) levels in NET patients before PRRT and after PRRT. The AUROC (blue curve) for differentiating NET patients before PRRT from NET patients after PRRT was 0.70 [95% confidence interval (CI): 0.59–0.82. $p < 0.01$]. A maximum AUC = 1 identifies an ideal (perfect) differentiation between these groups. The diagonal red line (AUC = 0.5) corresponds to chance discrimination. The VEGF-R1 AUC = 0.65 (blue curve) and $p < 0.01$ indicate that it is a good biomarker, and it can differentiate NET patients before PRRT from NET patients after PRRT*

poor predictive PRRT-response markers. The results are shown in Figure 1.

Discussion

PRRT therapy is most often the second line of treatment in patients with advanced, unresectable G1/G2 NETs with disease progression [2]. Nowadays, the response to PRRT therapy can be assessed using anatomical imaging, i.e. in tests such as computed tomography/magnetic resonance imaging (CT/MRI) (according to the RECIST 1.1 criteria), by scintigraphic tests (the use of [^{68}Ga] Ga-DOTA-TATE PET/CT is of particular importance), and assessment of the concentration of non-specific markers in the blood, such as CgA [26]. The [^{68}Ga] Ga-DOTA-TATE PET/CT scan shows the greatest sensitivity in detecting bone changes in contrast to anatomical imaging, but it is still unknown how to interpret changes in radiotracer uptake after treatment, because reduced tracer uptake may indicate a lower number of somatostatin receptors (SSTR) for various reasons (including disease progression or response to therapy) [26]). Moreover, due to the nature of NETs, which tend to grow slowly, it is not entirely clear whether the RECIST 1.1 scale is the appropriate parameter [26]. CgA is a commonly used biomarker in the clinical practice for monitoring patients with NETs, also treated with PRRT. However, this is a non-specific biomarker that can be influenced by many factors [27]. Evaluation of the multi-gene biomarker NETest in the blood of NETs has shown that it is significantly superior to CgA, and the use of predictive genes (PPQ) can accurately determine which patients will benefit from PRRT therapy and then monitor the disease, but the availability of this biomarker is currently limited [28]. Effective biomarkers are still being sought to assess the response to the treatment in NET patients [2].

The role of VEGF and VEGF-R has been confirmed in numerous studies in malignant tumours [11, 29], including NETs [5, 30–32]. According to Bates et al., epithelial-mesenchymal transition (EMT), a process that facilitates the progression of cancer in colon cancer, is associated with significant expression of VEGF and VEGF-R1, and blocking VEGF-R1 is associated with massive apoptosis in cells which were in EMT [14]. There are many reports in the literature on the correlation of angiogenic factors with metastatic disease in NETs [15]. Hansel et al. examined 19 primary well-differentiated pancreatic NETs and 7 liver metastases to determine the expression of VEGF-A and its

family member VEGF-C by immunolabeling analysis. The investigators showed that VEGF-C showed low to moderate expression in primary pancreatic NETs with significantly increased expression in liver metastases, while increased expression of VEGFR-2 and VEGFR-3 suggested a possible role in autocrine and paracrine tumourigenesis processes [33]. A summary of current research on the importance of angiogenic factors in GEP-NETs is presented in the work by Irina Sandra et al. [15]. According to Pavel et al. VEGF may correlate with disease progression in NETs [12]. Similarly, Berkovi´c et al. reported that VEGF is also increased in the case of GEP-NETs, especially hormonally active ones, and with lymph node metastases [34]. Similar observations were reported by the authors for VEGF-R1, which was increased in the setting of metastatic disease in NETs [13], and VEGFR-2 which may allow the prediction of overall survival (OS) in the case of pancreatic NETs [35]. In our study, we showed that in all patients included in the study in IV clinical stage, both VEGF-R1 and VEGF-R2 were increased before PRRT and significantly decreased after PRRT, while VEGF concentrations did not show statistically significant differences. Similarly, in some studies, no statistically significant differences were found between VEGF concentrations in the group of patients with NENs and in the control group [36]. As is known, tissue hypoxia associated with flow stasis in damaged vessels increases the concentration of VEGF, which is a factor promoting tumour growth and progression in malignant tumours [37]. It is worth mentioning, however, that paradoxically in the case of NETs, the angiogenesis seems to be independent of tissue hypoxia, and this phenomenon has been presented as the so-called "neuroendocrine paradox", in which highly differentiated NETs with a low degree of malignancy are characterised by the richest vascularisation, and therefore the density of the vascular network corresponds to the degree of differentiation rather than the degree of aggressiveness of the tumour [15]. Highly differentiated NETs can synthesise and constitutively secrete VEGF into the bloodstream, while in low-differentiated NETs this process is not constant [38].

Attempts to use the assessment of angiogenic factors in assessing treatment effectiveness have been studied for other therapies in NETs. In clinical practice, therapies currently used in NETs are related to angiogenesis pathways. In the first-line treatment of advanced or metastatic, slowly growing, well-differentiated G1/G2 NETs, somatostatin analogues (SSAs) are primarily used [1, 39]. SSAs have their place in the treatment of NETs due to their antiangiogenic effect directly through the presence of somatostatin receptors on endothelial cells, as well as indirectly by inhibiting the secretion of growth factors [40, 41]. However, Rosiek et al. showed that angiogenesis factors (VEGF and VEGF-R1) seem to have limited use in assessing the effectiveness of SSA treatment in NETs [42]. The study observed a decrease in VEGF concentration and an increase in VEGF-R1 concentration during treatment, while VEGF-R1 showed the best effectiveness in differentiating patients with NETs from healthy individuals [42]. Another study assessing the effectiveness of SSAs treatment in patients with NENs showed that the greatest decrease in VEGF-R2 occurred after 2 years of SSAs treatment, although, as the authors emphasise, the tested angiogenic factors (VEGF-R2, VEGF-R3 and vascular cell adhesion molecule 1 (VCAM-1) are not effective in monitoring patients treated with SSAs [43]. In our work, we confirmed that VEGF-R1 and VEGF-R2 decreased significantly after PRRT treatment, but only VEGF-R1 is a potential biomarker that can be used to assess the effectiveness of PRRT treatment. In the advanced stage of pancreatic NETs G1/G2 disease, 2 drugs with anti-angiogenic properties are also used: a selective m-TOR pathway inhibitor — everolimus, and a tyrosine kinase receptor inhibitor — sunitinib [44, 45]. In the randomised phase III RADIANT-3 clinical trial, everolimus treatment also led to a significant and progressive reduction in VEGF-R2 [45]. The role of sunitinib in the angiogenesis process has also been studied to assess the effectiveness of therapy and monitor patients [46]. Similarly, one study found that the mean plasma VEGF-R2 concentration was reduced after treatment [38, 47]. Likewise, in another study in NETs with metastatic disease, after 28 days of sunitinib administration, VEGFR-2 and VEGF-R3 levels decreased by ≥ 30% in approximately 60% and 70% of all patients, respectively, and returned to baseline values after 2 weeks of treatment break [38, 48]. It is not clear why VEGF-R3 increased after radioisotope therapy in our study. However, some authors emphasised that serum VEGF levels significantly correlated with VEGF-R3 in colorectal cancer [49]. Other authors confirmed that VEGF-R3 is not an effective marker in assessing patients treated with SSAs in NETs [43].

Considering the complexity and heterogeneity of NETs treated with PRRT [50], the importance of molecular imaging phenotyping for effective PRRT therapy and an individual approach to treatment is emphasised [51]. Gianetta et al. report that the inflammatory process associated with tumour-associated neutrophils (TAN) promotes the disease progression through the high expression of pro-angiogenic factors, such as VEGF [38]. Ohlendorf et al. also tried to assess whether cancer-related inflammatory markers might play a role in patients with GEP-NETs treated with PRRT and showed that, although these parameters showed

significant heterogeneity, they were higher in patients not responding to PRRT therapy [52].

There are no data in the literature regarding the assessment of angiogenic factors in patients treated with PRRT. In our opinion, further prospective studies are still needed to precisely assess these parameters in a larger group of NET patients.

Conclusions

Although the concentrations of CgA, VEGF-R1, and VEGF-R2 decreased significantly after PRRT therapy, only VEGF-R1 is a potential biomarker in assessing the effectiveness of PRRT treatment. In the case of progressive patients with NETs undergoing PRRT treatment, the assessment of angiogenic factors seems important, but further prospective studies are still needed to precisely assess these parameters in NET patients.

Advantages of the study

According to the available literature (PubMed Database), this is one of the first studies evaluating angiogenic factors in NET patients treated with PRRT.

Limitations of the study

This study has potential limitations: it is limited by the small number of patients and limited follow-up time.

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Author contributions
Conceptualisation, J.S, K.M.; Methodology, J.S, M.W.-G., V.R. Formal Analysis, J.S., V.R.; Investigation, J.S., M.W.-G., K.M.; Resources, J.S., K.M., M.W.G., Writing — Original Draft Preparation, J.S; M.W.-G., K.M., Writing — Review & Editing, V.R., G.K., D.K., B.K-K.; Visualization, M.W-G., V.R.; Supervision, B.K.-K.; Project Administration, J.S.

Institutional Review Board Statement
The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Medical University of Silesia (approval codes: PCN/022/KB1/97/I/II/19/20). All patients and controls signed the informed consent.

Informed consent statement
Informed consent was obtained from all subjects involved in the study.

Data availability statement
The data used to support the findings of this research are available upon request from the corresponding author, Janusz Strzelczyk: janusz.strzelczyk@sum.edu.pl.

Conflicts of interest
The Authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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