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Association between preoperative Vasostatin-1 and pathological features of aggressiveness in localized nonfunctioning pancreatic neuroendocrine tumors (NF-PanNET)

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ABSTRACT

Background: A reliable and accessible biomarker for nonfunctioning pancreatic neuroendocrine tumors (NF-PanNET) is currently unavailable. Chromogranin A (CgA) represents the best-described neuroendocrine biomarker, but its accuracy is low. Vasostatin-1 (VS-1), a fragment derived from the cleavage of CgA, was recently investigated and found to be more accurate as tumor biomarker in a cohort of patients affected by mainly metastatic small intestinal NET.

Methods: Patients submitted to surgery for sporadic localized NF-PanNET at San Raffaele Hospital were included. Preoperative plasma samples were prospectively collected. Circulating levels of total-CgA and VS-1 were retrospectively investigated by sandwich Enzyme-Linked ImmunoSorbent Assays.

Results: Overall, 50 patients were included. VS-1 value (P=0.0001) was the only preoperatively retrievable factor independently associated with NF-PanNET size. No significant correlation between CgA and tumor diameter was found ($P = 0.057$). A VS-1 value of 0.39 nM was identified as the optimal VS-1 cut-off accurately associated with NF-PanNET larger than 4 cm. Patients with VS-1 > 0.39 nM had a significantly higher frequency of microvascular invasion ($P = 0.005$) and nodal metastasis ($P = 0.027$). Median VS-1 plasma level was significantly higher in the presence of microvascular invasion ($P = 0.001$) and nodal metastasis ($P = 0.012$). PPI assumption significantly increased total-CgA levels, but not those of VS-1 ($P = 0.111$).

Conclusions: In localized, non-metastatic NF-PanNET, VS-1 is strongly associated to tumor dimension and its plasma levels are significantly higher in the presence of microvascular invasion and nodal metastases; moreover, VS-1 value is not affected by the PPI use.

Introduction

Pancreatic neuroendocrine tumors (PanNET) have been traditionally considered as rare lesions, accounting for approximately 2% of all pancreatic tumors [1]. Despite still being still regarded as uncommon neoplasms, their incidence has significantly increased during the last two decades, raising from 0.4 per 100,000 inhabitants in 2002 to 0.8 per 100,000 inhabitants in 2012 [2].

To date, the lack of a reliable and accessible neuroendocrine biomarker has represented a crucial unmet clinical need, especially in the case of localized nonfunctioning (NF) PanNET. Chromogranin A (CgA) currently represents the most practical and useful biomarker for these lesions [3]. Nevertheless, CgA has shown low accuracy in terms of sensitivity, specificity and standardization of assays. CgA sensitivity varies from 60% to 100% [4], as its plasma levels are tightly related to tumor hormone-secretion and disease extent. CgA, in fact, can be considered an acceptable biomarker for functional and advanced PanNET, but it is extremely poor for localized nonfunctioning disease [5]. A further limitation is that

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circulating CgA levels are affected by several conditions such as proton pump inhibitors (PPI) consumption, atrophic gastritis, impaired kidney function, chronic heart failure, acute coronary syndromes, hypertension, autoimmune diseases, atherosclerosis and non-neuroendocrine neoplasms [6–13].

A recent study [14] investigated the circulating levels of fragments derived from the proteolytic cleavage of CgA. Among these peptides, plasma levels of Vasostatin-1 ($CgA_{1\text{-}76}$) were found to be significantly increased in patients with ileal and pancreatic NET as compared to healthy subjects and were not affected by PPI therapy. Nevertheless, this prior report [14] included a heterogenous cohort of patients mainly affected by metastatic, functioning, small intestinal NET (si-NET). Therefore, possible correlation between VS-1 and localized nonfunctioning (NF)-PanNET is still unknown.

The aim of the present study was to assess a possible association between preoperative VS-1 plasma levels and pathological features of aggressiveness, in comparison to CgA, in a cohort of patients affected by sporadic localized NF-PanNET.

Methods

This study was conducted according to the "Standards for Reporting Diagnostic accuracy studies" guidelines (STARD 2015) [15].

Preoperative plasma samples of candidates for surgical resection for PanNEN at San Raffaele Scientific Institute were prospectively collected from May 2015. Written informed consent was obtained from all the subjects and the study was approved by the San Raffaele Ethics Committee (protocol NETBANK001). All the patients diagnosed with asymptomatic NF-PanNET > 2 cm underwent surgery, whereas those with asymptomatic NF-PanNET ≤ 2 cm without signs of aggressiveness (dilation of main pancreatic duct or bile duct) were conservatively managed. NF-PanNET were defined as not resectable in the presence of superior mesenteric artery involvement and/or in the presence of a portal vein/superior mesenteric infiltration >180°. For the purpose of the present research, only patients submitted to surgery between May 2015 and 2017 for nonfunctioning, sporadic PanNET G1-G2-G3 were considered. Patients diagnosed with functioning forms or with metastatic disease were excluded, as well as those with tumors classified as pancreatic neuroendocrine carcinoma (PanNEC) G3 [16].

VS-1 ($CgA_{1\text{-}76}$) represented the index test, whereas total-CgA was chosen as reference standard test. VS-1 and total-CgA were detected in the plasma samples using two sandwich Enzyme-Linked ImmunoSorbent Assays (ELISA), called *76- and "total-CgA"- ELISA [14,17], respectively. *76-ELISA can selectively detect the N-terminal fragment $CgA_{1\text{-}76}$, whereas "total-CgA"- ELISA is able to detect full-length CgA plus fragments containing the N-terminal and all or part of the central and C-terminal regions. The *76-ELISA was based on the use of mouse monoclonal antibody (mAb) 5A8 (epitope $CgA_{53\text{-}57}$) and rabbit polyclonal antibody α76 (epitope $CgA_{71\text{-}76}$) as capturing and detecting antibodies; total-CgA-ELISA was based on the use of mAb B4E11 (epitope $CgA_{68\text{-}71}$) and rabbit polyclonal αCgA (FRs). Different monoclonal antibodies (5A8 and B4E11) were used as capturing antibodies in the two assays because mAB B4E11 and α76 could not form molecular sandwiches, due to steric hindrance. The polyclonal antisera α76 was raised in a rabbit by immunization with the peptide $CgA_{71\text{-}76}$, whereas the polyclonal antisera αCgA (FRs) was raised in rabbit by immunization with recombinant human CgA.

Results of the index test were available to the assessors of the reference standard test and viceversa, whereas clinical information was not accessible to performers and readers of both index and reference standard tests. Blood was collected in EDTA-containing tubes and plasma was obtained after centrifugation of each sample (1600g for 10 min, +4 °C). Plasma samples were frozen slowly (−1 °C per minute), then stored for 24–72 h in a −80 °C refrigerator, and finally transferred into liquid nitrogen.

A prospectively collected database was queried for clinical and pathological details. The preoperative variables considered were gender, age, presence of symptoms, body mass index (BMI), PPI use, VS-1 and total-CgA plasma levels. Tumor size was defined as the largest diameter measured in the pathological specimen. T and N stages were classified according to the current AJCC [18] and ENETS [19] classifications. Immunostaining routinely included synaptophysin, CgA and Ki67 proliferative index, assessed by MIB1 antibody staining and expressed as the percentage of cells with positive nuclear staining in 2000 cells counted in the area of highest nuclear labelling. Tumor grade was classified according to the 2017 WHO classification [16] into G1 (Ki67 index < 3%), G2 (Ki67 index 3–20%) and G3 (Ki67 index > 20%). Surgical margins were distinguished into three categories: R0 (no residual tumor), R1 (microscopic residual tumor), R2 (macroscopic residual tumor). The presence of microvascular invasion, perineural invasion and necrosis was assessed. A Recurrence Risk Score based on tumor grade, presence of lymph node metastasis and perineural invasion was calculated for each patient according to *Genc* et al. [20].

All the patients included in the study were followed-up regularly after surgery, but disease specific survival was not considered as possible endpoint due to short period of follow-up.

Statistical analysis

Continuous data were reported as median and interquartile range (IQR). For categorical data, number and proportion (%) were displayed. The comparison between subgroups was carried out using the Mann-Whitney *U* test for continuous variables. Qualitative data were compared by the Chi square test. The relationship between continuous variables was evaluated by linear regression analyses. The correlation with tumor diameter was assessed in order to find a VS-1 cut-off for stratifying the cohort and comparing pathological features of aggressiveness between patients with different values of VS-1. Receiver Operating Characteristics (ROC) analysis was performed to determine the accuracy and the most suitable cut-off for index and reference tests and their association with tumor size, nodal metastases and microvascular invasion. Statistical analyses were performed in SPSS 16.0 for Windows software (SPSS Inc, Chicago, Illinois, USA). *P* values were considered significant when less than or equal to 0.05. There were no indeterminate or missing data for either VS-1 or total-CgA.

Results

Participants

Overall, 50 patients submitted to pancreatic resection for sporadic localized NF-PanNET were included in the study. Demographics, clinical details and pathological findings are summarized in Table 1.

Test results

By linear regression analysis, the only preoperatively retrievable factor significantly correlated with tumor size was VS-1 value ($B = 38.336$, 95%CI: 28.492; 48.179, $P=0.0001$). No correlation between total-CgA value ($B = 3.188$, 95%CI: - 0.096; 6.471, $P=0.057$) and tumor diameter was found. ROC curves were performed in order to evaluate the accuracy of VS-1 and total-CgA and their association with tumor size (Table 2). Global performance, expressed

Table 1
Demographics, perioperative characteristics and histological findings of 50 patients with histologically proven nonfunctioning pancreatic neuroendocrine tumors (NF-PanNET).

Variable	*n (%)*
Gender	
Male	26 (52)
Female	24 (48)
Age, years[a]	58 (49; 64)
Pain	
No	37 (74)
Yes	13 (26)
BMI, kg/m^2[a]	24.5 (21.9; 27.6)
PPI use	
No	39 (78)
Yes	11 (22)
Chromogranin A, nM[a]	0.664 (0.490; 1.608)
Vasostatin-1, nM[a]	0.340 (0.305; 0.446)
Largest diameter, mm[a]	25 (18; 37)
Ki67, %[a]	3 (1; 6.5)
Grade [16]	
G1	20 (40)
G2	28 (56)
G3	2 (4)
T stage	
T1	16 (32)
T2	23 (46)
T3	11 (22)
N stage	
N0	31 (62)
N1	19 (38)
Stage	
I	14 (28)
II	17 (34)
III	19 (38)
Resection margins	
R0	45 (90)
R1	4 (8)
R2	1 (2)
Microvascular invasion	
No	28 (56)
Yes	22 (44)
Perineural invasion	
No	39 (78)
Yes	11 (22)
Necrosis	
No	46 (92)
Yes	4 (8)
Recurrence Risk Score [20] [b]	
0	16 (33)
24	2 (4)
40	12 (25)
48	2 (4)
64	12 (25)
88	4 (9)

BMI: Body Mass Index.
PPI: Proton Pump Inhibitors.
[a] Expressed as median (IQR).
[b] Applicable only to PanNET G1-G2.

as area under the curve (AUC), was 0.872 ($P < 0.0001$) for VS-1 using a cut-off of 4 cm compared to 0.764 ($P = 0.003$) when using a cut-off of 2 cm. The correlation between total-CgA values and NF-PanNET size was significant only when using a cut-off of 2 cm (AUC 0.699, $P = 0.025$), whereas it was not when a 4 cm cut-off was considered (AUC 0.648, $P = 0.152$). The optimal VS-1 cut-off accurately associated with a NF-PanNET larger than 4 cm was 0.39 nM with a sensitivity of 80% and a specificity of 80%.

The study cohort was then stratified according to the VS-1 cut-off previously identified. Comparison of patients with VS-1 $\leq$ 0.39 nM and VS-1 > 0.39 nM is depicted in Table 3. The two groups were similar for gender, age, PPI use, tumor grade, recurrence risk score, presence of necrosis and perineural invasion. Patients with a

Table 3
Comparison of clinical and pathological features according to the Vasostatin-1 (VS-1) value $\leq$ 0.39 nM ($n = 34$) and VS-1 value > 0.39 nM ($n = 16$).

Variable	VS-1 $\leq$ 0.39 nM, *n* (%)	VS-1 > 0.39 nM, *n* (%)	*P*
Gender			
Male	20 (59)	6 (37)	
Female	14 (41)	10 (63)	0.227
Age			
> 60 years	19 (56)	9 (56)	
$\leq$ 60 years	15 (44)	7 (44)	1.000
Pain			
No	26 (76)	11 (69)	
Yes	8 (24)	5 (31)	0.731
BMI			
> 25 kg/m^2	16 (47)	11 (69)	
$\leq$ 25 kg/m^2	18 (53)	5 (31)	0.225
PPI			
No	28 (82)	11 (69)	
Yes	6 (18)	5 (31)	0.297
Tumor Grade [16]			
G1	17 (50)	5 (19)	
G2	16 (47)	12 (75)	
G3	1 (3)	1 (6)	0.074
N stage			
N0	25 (74)	6 (37)	
N1	9 (26)	10 (63)	**0.027**
Microvascular invasion			
No	24 (71)	4 (25)	
Yes	10 (29)	12 (75)	**0.005**
Necrosis			
No	32 (94)	14 (88)	
Yes	2 (6)	2 (12)	0.584
Perineural invasion			
No	27 (79)	12 (75)	
Yes	7 (21)	4 (25)	0.728
Recurrence Risk Score [20] [a]			
> 40	21 (70)	9 (50)	
$\leq$ 40	9 (30)	9 (50)	0.166

BMI: Body Mass Index.
PPI: Proton Pump Inhibitors.
[a] Applicable only to PanNET G1-G2.

Table 2
Association between Vasostatin-1 (VS-1), Chromogranin-A and NF-PanNET size according to different cut-offs.

Vasostatin-1 (VS-1)					
Tumor size cut-off	**AUC**	***P***	**VS-1 value (nM)**	**Sensitivity (%)**	**Specificity (%)**
> 2 cm	0.764	0.003	0.32	82	56
> 4 cm	0.872	<0.0001	0.39	80	80
Chromogranin-A (CgA)					
Tumor size cut-off	**AUC**	***P***	**CgA value (nM)**	**Sensitivity (%)**	**Specificity (%)**
> 2 cm	0.670	0.025	0.59	65	56
> 4 cm	0.648	0.152	–	–	–

AUC: Area Under the Curve.

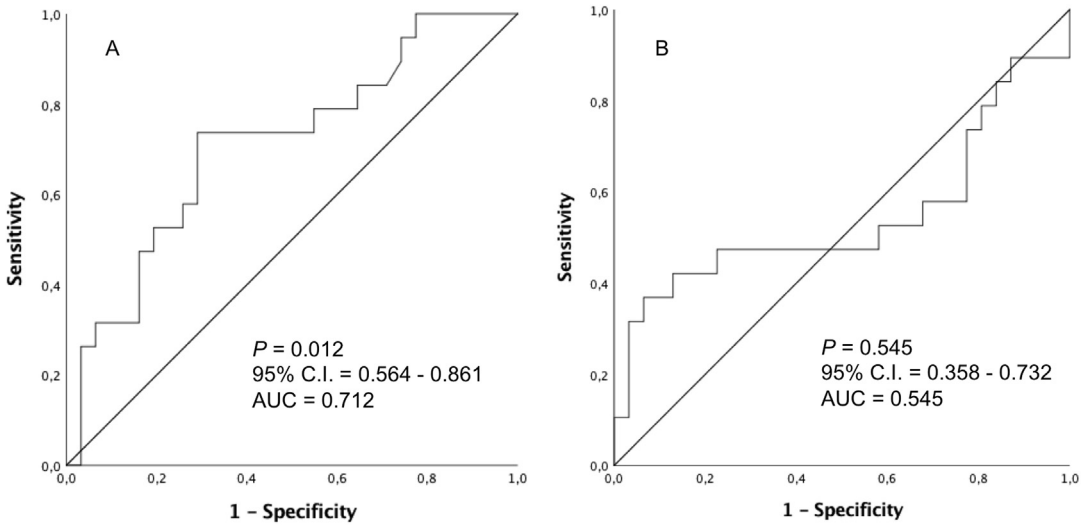

Fig. 1. Receiver Operating Characteristic (ROC) curves of Vasostatin-1 (**1A**) and total-CgA (**1B**) as predictors of lymph node metastases.

VS-1 value > 0.39 nM had a significantly higher frequency of nodal metastases ($P = 0.027$) and microvascular invasion ($P = 0.005$).

ROC curves were performed in order to evaluate the accuracy of VS-1, compared to total-CgA, in predicting the presence of nodal involvement and microvascular invasion. Global performances of VS-1, expressed as AUC, were 0.712 ($P = 0.012$) for identifying the presence of lymph node metastases (Fig. 1A) and 0.763 (Fig. 2A) for predicting the presence of microvascular invasion. Global performances (AUC) of total-CgA were 0.545 ($P = 0.596$) (Figs. 1B) and 0.594 ($P = 0.257$) (Fig. 2B) for predicting lymph node metastases and microvascular invasion, respectively.

Median VS-1 plasma level was significantly higher in the presence of microvascular invasion [0.411 nM (IQR 0.324–0.671) vs 0.326 nM (IQR 0.296–0.355), P=0.001] and nodal metastases [0.395 nM (IQR 0.324–0.655) vs 0.327 nM (IQR 0.294–0.375), P=0.012] (Fig. 3AandB). Patients assuming PPI had a significantly increased circulating total-CgA compared to the remaining patients [1.799 nM (IQR 1.417–3.968) vs 0.591 nM (0.443–0.908); P<0.0001], whereas VS-1 levels were similar between these two groups [0.386 nM (IQR 0.328–0.791) vs 0.327 nM (0.302–0.396), P=0.111]. No significant correlation was found between total-CgA and VS-1 (r = 0.052, P=0.119).

Discussion

The present study demonstrated that preoperative VS-1 plasma levels correlate with tumor diameter and with some pathological features of aggressiveness in patients who were candidates for surgery for localized NF-PanNET. In contrast, no correlation between preoperative total-CgA plasma levels, tumor size and pathological features of aggressiveness was found.

Diagnostic accuracy of biomarkers for neuroendocrine tumors has been rarely evaluated in a selected population of patients with well-differentiated, localized and resectable NF-PanNET. Regarding CgA, only *Jilesen* et al. [21] considered a cohort of patients affected by non-metastatic, NF-PanNET G1-G2 observing that preoperative CgA elevation was present in only one third of them. On the contrary, several studies [22–26] investigated the role of CgA in heterogeneous cohorts with regards to the stage and the function of the lesions. *Nolting and coworkers* [22] showed that CgA values

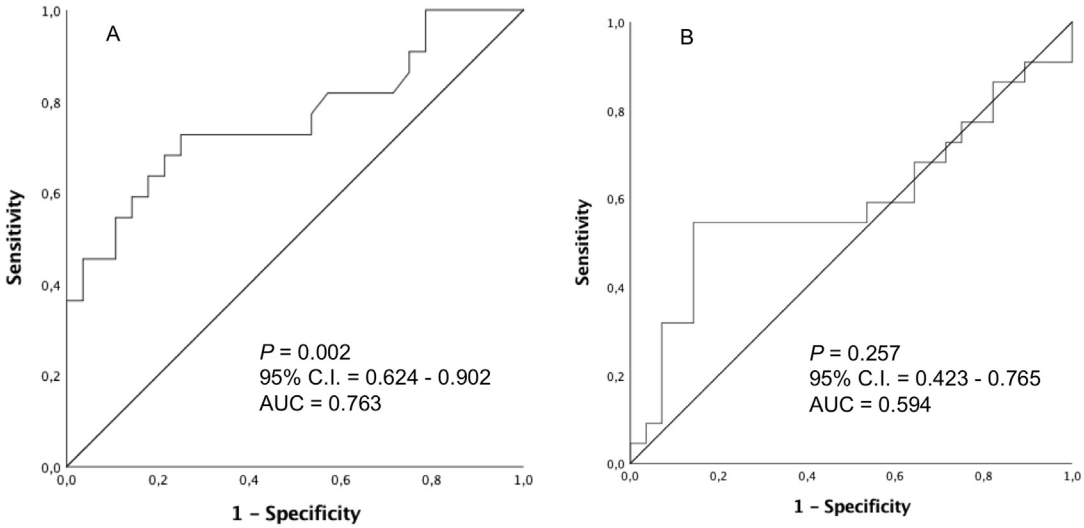

Fig. 2. Receiver Operating Characteristic (ROC) curves of Vasostatin-1 (**2A**) and total-CgA (**2B**) as predictors of microvascular invasion.

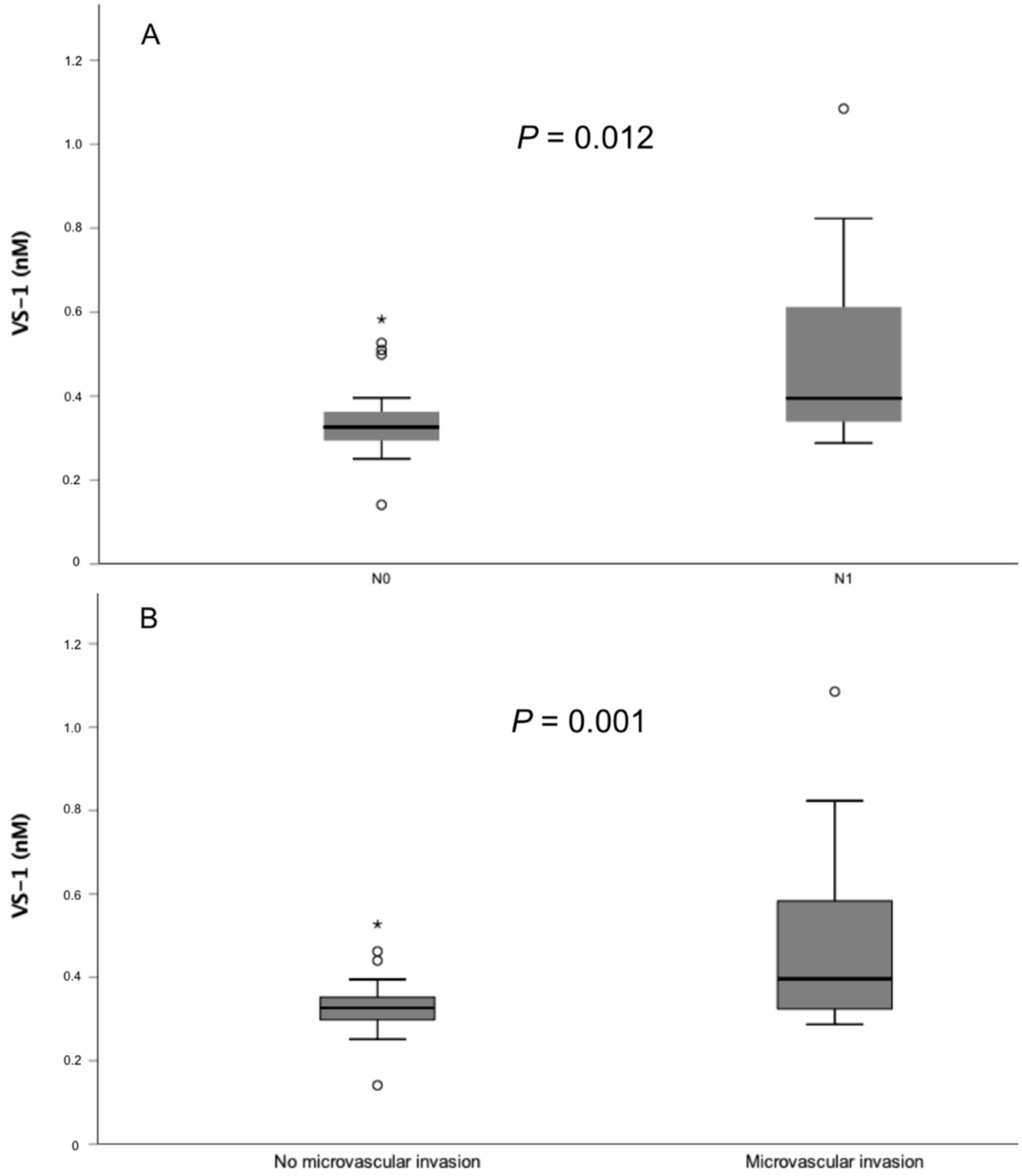

Fig. 3. Plasma levels of Vasostatin-1 (VS-1) in patients with and without microvascular invasion (**3A**) and with and without nodal metastases (**3B**). Box-plots with median (middle line), 75th percentile (top line) and 25th percentile (bottom line). ◦ represents outliers and * represents extreme outliers.

were significantly higher in patients with liver metastases when compared to those with localized disease. Similar results were confirmed by two other studies [24,27]. The presence of distant metastases was not the only factor associated with an elevated level of CgA. In fact, the presence of functioning forms and a small intestinal origin of the tumor represent two additional elements, which are strictly associated with increased values of CgA. Two prior series demonstrated that the presence of small intestinal NET (si-NET), especially when functioning, was associated with higher CgA levels if compared to PanNET [22,28]. Consistently, *Nehar* et al. [29] reported that circulating plasma levels of CgA were significantly increased in patients with functioning tumors. Therefore, in the presence of a localized NF-PanNET, the preoperative determination of CgA seems to be inaccurate and its routinely use is not recommended [21–23,26].

Nodal involvement and NF-PanNET dimension are powerful prognostic factors for these lesions. Some of the authors of the present study previously demonstrated that the risk of disease recurrence after surgery for patients affected by PanNET with nodal metastases (N+) is significantly higher compared to that of patients without nodal involvement [30,31]. These results were consistent with a prior study by *Hashim* et al. [32] reporting poorer survival

rates for patients with nodal metastases. Moreover, it is hard to preoperatively identify those patients with N+ neoplasms. In the present series, higher VS-1 plasma levels were associated with the presence of lymph node metastases. This result could be clinically relevant if validated with a larger sample size, as the possibility of predicting nodal involvement before surgery would be useful in order to choose the appropriate type of resection and lymphadenectomy.

Moreover, larger NF-PanNET are more likely to be associated with features of aggressiveness and risk of disease recurrence after surgery [33]. In the setting of localized, non-metastatic disease, the largest tumor diameter represents then an important measure of the disease burden. In the current series, VS-1 plasma levels were strictly associated with tumor size, showing the highest accuracy for NF-PanNET > 4 cm. This result suggests that the higher is the tumor burden, the higher is the production of VS-1 by the NF-PanNET. Among all the possible biomarkers evaluated so far, a correlation between tumor size and circulating plasma levels has only been assessed for CgA. Consistently with the results of the present series, *Jilesen* et al. [21] found no correlation between preoperative CgA values and tumor diameter in patients affected by resectable NF-PanNET. Similar results were reported by two other series [24,25] comprising a more heterogeneous population. In contrast, *Jun* et al. [34] recently observed a significant correlation between PanNEN diameter and CgA value. Nevertheless, it should be pointed out that all the patients with a history of PPI inhibitors assumption, malignant tumor, renal and/or cardiac insufficiency, were excluded from this study.

The assumption of PPI inhibitors undoubtedly represents one of the main determinants of CgA value increasing [27]. Remarkably, the present experience showed that the administration of PPI increased total-CgA plasma levels, leaving VS-1 values unchanged.

These results corroborate those reported in the only series published so far on a possible role of VS-1 as neuroendocrine biomarker [14]. In contrast to the finding presented by *Corsello* et al. [14], no correlation between preoperative VS-1 and total-CgA plasma levels was found in the current study. A possible explanation is that the cohort of patients considered in the prior series was heterogeneous in terms of stage and site of origin of the tumors. This difference may also explain the lower levels of both total-CgA and VS-1 measured in the present series when compared with those previously reported [14]. With regard to other biomarkers, the NETest is gaining wide acceptance as a possible novel neuroendocrine biomarker [23]. NETest is a blood-based multianalyte-derived NET gene signature encompassing the expression of 51 genes, assessed by 4 different prediction algorithms and then scaled to a disease/tumor activity (0–100%) score [35]. Since current evidence seems to support the role of multianalyte tests for diagnosis and prediction of NET progression, it could be argued that further researches on possible novel biomarkers may not be useful. Nevertheless, VS-1 may have the advantage of being readily available, reproducible as well as cost-effective. This is even more important considering the long-life expectancy of patients affected by localized NF-PanNET as well as the long median time needed for either follow up or detection of eventual recurrences.

The present study has several limitations that need to be recognized. The most important is that possible clinical implications of the present study are limited. Although high levels of VS-1 were associated with pathological features of aggressiveness of NF-PanNET, such as nodal involvement and microvascular invasion, this finding was probably related to the association observed between VS-1 and tumor size. A second limitation concerns the small sample size that did not allow to evaluate the effect of possible confounding factors. Thirdly, VS-1 and total-CgA levels were measured only preoperatively. Consequently, it was not possible to determine the value of VS-1 in terms of surgical efficacy and presence of possible residual disease. Finally, the post-operative follow-up was too short to evaluate a possible role of VS-1 levels in predicting the risk of disease recurrence after surgery. Conversely, the study also has the strength of providing interesting preliminary results, which render VS-1 as a promising biomarker for PanNEN. Moreover, inclusion criteria were clearly defined, allowing to select a cohort of patients affected by homogenous lesions, thus considerably reducing the risk of bias.

In conclusion, VS-1 is strongly associated with tumor dimension and some pathological features of aggressiveness in localized NF-PanNET; moreover, it is not affected by PPI therapy. The current study supports the hypothesis that VS-1 represents a promising biomarker, which needs to be studied in future prospective trials.

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Disclosure statement

The authors declare that there is no conflict of interest associated with this publication.

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