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ORIGINAL ARTICLE

Vitamin D deficiency and tumor aggressiveness in gastroenteropancreatic neuroendocrine tumors

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Abstract

Purpose Data regarding vitamin D status in patients affected by gastroenteropancreatic (GEP) neuroendocrine tumor (NET) are limited and often showing contrasting results. The aim of the study was to evaluate the incidence of vitamin D deficiency (<20 ng/mL) in GEP-NET patients and compare the 25-hydroxyvitamin D (25(OH)D) levels with clinicopathological parameters and clinical outcome.

Methods A retrospective cross-sectional study including 75 low grade (G1-G2) GEP-NETs and 123 healthy controls matched for age, sex, and body mass index, was performed.

Results GEP-NET patients had significantly lower 25(OH)D levels compared to controls (17.9 ± 7.8 vs 24.2 ± 7.7 ng/mL, $p < 0.0001$). Ileal NETs were associated to lower 25(OH)D levels compared to other primary tumor sites ($p = 0.049$) and small bowel resection posed a significant increased risk of severe vitamin D deficiency (OR = 2.81, 95% CI = 1.25–3.37, $p = 0.018$). No correlation with somatostatin analogs treatment was found. 25(OH)D levels were significantly lower in G2 compared to G1 GEP-NETs (15.6 ± 7.8 vs 19.9 ± 7.4 ng/mL, $p = 0.016$) and in patients with progressive disease (12.6 ± 5.7 ng/mL) compared to those with stable disease (mean 21.5 ± 8.2 ng/mL, $p = 0.001$) or tumor free after surgery (19.6 ± 7.3 ng/mL, $p = 0.002$). Patients with vitamin D deficiency and insufficiency had shorter progression-free survival compared to those with sufficiency ($p = 0.014$), whereas no correlation was found with disease-specific survival.

Conclusions Vitamin D deficiency is highly prevalent among GEP-NETs and could be associated with high tumor grade and disease progression. Therefore, the monitoring of 25(OH)D levels is relevant in these patients and vitamin D supplementation should be considered in the management of GEP-NET patients with vitamin D deficiency or insufficiency.

Keywords Neuroendocrine tumor · Vitamin D · 25(OH)D · PTH · MEN1 · Somatostatin analogs · Prognosis

Introduction

The pleiotropic effect of vitamin D is largely described in the literature [1]. Due to the expression of vitamin D-activating enzymes and receptors in different cell types, vitamin D plays a role in the pathogenesis and outcome of several clinical conditions, including diabetes, cardiovascular diseases, autoimmune diseases, as well as different cancer types [2–5]. Current evidence suggests that circulating levels of 25-hydroxyvitamin D [25(OH)D] above 21.6 ng/mL (54 nmol/L) and above 30 ng/mL (75 nmol/L) may contribute to reduce cancer mortality and cancer risk, respectively [6, 7].

Neuroendocrine neoplasms (NENs) comprise a heterogeneous group of relative rare neoplasms arising from the diffuse neuroendocrine cell system, mostly occurring in the

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gastroenteropancreatic (GEP) tract. Most GEP-NENs are well-differentiate neuroendocrine tumors (NETs) and occur sporadically, although they may arise in the setting of hereditary syndromes, such as the multiple endocrine neoplasia type 1 (MEN1). In MEN1, GEP-NETs are usually multifocal and are characterized by an early age of onset [8]. Compared to patients with other types of solid tumors, GEP-NET patients have generally a long-term survival also in case of advanced disease [9, 10], and usually present several comorbidities [10–12], including a high risk of vitamin D deficiency [13]. Depending on the definition used, vitamin D deficiency has been described in a range between 31% and 68% of GEP-NETs [14–17]. This increased risk seems to be mostly related to a condition of malnutrition caused by tumor hormonal hypersecretion, surgical tumor resection, which modifies the anatomy of the gastrointestinal tract, and treatment with somatostatin analogs (SSAs), which inhibits the gastrointestinal and pancreatic exocrine and the endocrine secretion [18]. All these components could affect the gut's secretion and motility, with consequent diarrhea and steatorrhea, leading to malabsorption of nutrients [19, 20]. The first studies investigating the 25(OH)D levels in patients with NEN evaluated relatively small or heterogeneous cohort of patients [14, 15, 21]. The two more recent studies [16, 17], although evaluated a larger cohort of GEP-NET patients, reported contrasting results in the impact of SSAs on 25(OH)D levels and in the correlation between vitamin D status and clinical outcome. Nevertheless, both studies demonstrated that vitamin D supplementation improved 25(OH)D levels in most GEP-NET patients [16, 17]. However, it remains unclear if patients with MEN1 and primary hyperparathyroidism (PHPT) were included in these previous studies. Due to the impact of PHPT on vitamin D metabolisms [13, 22], the inclusion of patients with PHPT could create a bias in the evaluation of the vitamin D status in GEP-NETs.

These discrepancies suggest the necessity to investigate the role of vitamin D in a well-characterized cohort of NET patients. Therefore, the aim of this study was to evaluate 25(OH)D levels in GEP-NET patients with well-differentiated, low-grade tumors, in comparison to a matched healthy control group, and to correlate 25(OH)D levels with parameter of tumor aggressiveness and clinical outcome.

Materials and methods

Study design and population

We performed a retrospective cross-sectional study including GEP-NET patients and healthy controls matched by age, sex, and body mass index (BMI). Both patients and controls were vitamin D-treatment naive. Blood samples for the 25(OH)D evaluation were collected the between winter and spring seasons (November–April, starting from 2007) to rule out seasonal influences on vitamin D levels [23]. For NET patients, blood samples were collected at the first or second admission to our department. NET patients and controls were from the geographical area around the Naples metropolitan area in Italy (latitude 40° 49′ N; elevation 17 m). We included only patients with well-differentiated, low-grade G1 and G2 GEP-NET. Among MEN1 patients, we excluded those with PHPT, to avoid any potential interference with vitamin D status. Other exclusion criteria were age <18 years old, high-grade G3 GEP-NET or neuroendocrine carcinoma, chronic use of medicaments or supplements known to interfere with vitamin D metabolism in the last 3 months (including cinacalcet, calcium, anti-osteoporosis medicaments, sex-hormones, anti-inflammatory), liver or renal insufficiency, acute or chronic inflammatory diseases. Controls were recruited from healthy volunteers from the hospital and employees with a negative history of cancer, diabetes mellitus, hypertension, liver or renal failure, inflammatory disease, and chronic use of medicaments.

The study was carried out at the European Neuroendocrine Tumor Society (ENETS) Center of Excellence at Unit of Endocrinology, Federico II University of Naples, Italy. Anthropometric measurements and biochemical assessment of both GEP-NET patients and healthy controls, as well as the disease status for the GEP-NET patients, were evaluated at the time of the blood collection. Last follow-up was December 2019. The study was approved by the local Ethical Committee (n. 201/17) and was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from both patients and healthy subjects.

Power analysis

The power justification was retrospectively calculated by the differences of means + standard deviation (SD) of 25(OH)D levels of the two studied groups (17.9 ± 7.8 vs 24.2 ± 7.7 in GEP-NET patients and control group, respectively). Considering a power size of 95%, with a type I (alpha) error of 0.01 (95%), and a type II (beta) of 0.05, the minimum number of cases required to detect a significant difference between the two groups was of 40 individuals for each group. The calculation of power size was performed using Sample Size Calculator Clinical Calc (https://clincalc.com/stats/samplesize.aspx), as previously reported [24].

Clinical and pathological characteristics of GEP-NET

Clinicopathological characteristics, such as sex, primary tumor site and size, tumor stage, Ki67 index, hormonal secretion, familiar history and genetic diagnosis of MEN1, treatment, and follow-up, were collected for all GEP-NET patients.

Most patients had a diagnosis of NET within the 2 years before the collection of the blood sample used for the evaluation of the 25(OH)D. Histological diagnosis of GEP-NET was achieved after tumor resection or liver biopsy. In MEN1 patients, the diagnosis of pancreatic NET was achieved cytologically after endoscopic ultrasonography-guided fine-needle aspiration (FNA) or based on radiological evidence of the tumor in case of inconclusive FNA [25]. Tumor grade was classified according to the World Health Organization (WHO) 2010 classification [26] as G1 (Ki67% ≤2%) or G2 (Ki67% 3–20%).

Anthropometric measurements

Anthropometric measurements and age were evaluated both in NET patients and in the control group at the time of the 25(OH)D evaluation. Height, weight, BMI, and waist circumference (WC) were evaluated following standard criteria as previously reported [27–29]. BMI was classified according to WHO criteria as underweight (BMI < 18.5 kg/m^2), normal weight (BMI 18.5–24.9 kg/m^2), overweight (BMI 25.0–29.9 kg/m^2), and obesity (BMI ≥ 30.0 kg/m^2) [30].

Biochemical assessment

In all subjects, blood samples were collected in the morning between 8 and 10 a.m., after an overnight fast of at least 8 h, and stored at −80° until being processed. Serum Ca, phosphorus, and albumin were determined by automated techniques (Roche Modular System, Basel, Switzerland). Total Ca corrected for serum albumin was calculated using the following formula: corrected Ca (mg/dL) = measured total Ca (mg/dL) + 0.8 × (4.0 − serum albumin [g/dL]), where 4.0 represents the average albumin and 0.8 the correction factor [31]. Normal serum phosphorus range was 2.5–4.5 mg/dL. Intact parathyroid hormone (PTH) was measured by immunometric assay (Immulite iPTH from Diagnostics Products Corporation, Los Angeles, CA, US). Normal range was 16–87 pg/mL; intra- and interassay coefficients of variation were <7% and <9%, respectively [32]. The 25(OH)D levels were quantified by a commercial competitive chemiluminescence immunoassay (DiaSorin Liaison, Saluggia, Italy), which has a bias of 9.9% and 7.1% at target concentration of 20–40 and 50–70 nmol/L, respectively [33]. According to the Vitamin D External Quality Assurance Scheme (DEQAS) program, automated immunoassays with <10% bias can be used safely in clinical practice (www.deqas.com) [33]. In line with the Endocrine Society guidelines [34], 25(OH)D deficiency was defined as serum concentration of 25(OH)D < 20 ng/mL (50 nmol/L), insufficiency as levels between 20–30 ng/mL (50–75 nmol/L) and normal levels for values ≥30 ng/mL (≥75 nmol/L). Moreover, 25(OH)D levels <10 ng/mL (<25 nmol/L) were considered as severe deficiency [14].

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD), whereas categorical variables as numbers and percentages. Data distribution was evaluated by the Shapiro–Wilk test. Two-sided *t* test or ANOVA followed by Bonferroni *post-hoc* test and Mann–Whitney test or Kruskal–Wallis test followed by Dunn's *post-hoc* test was used to compare variables, as appropriate. Fisher's exact test or the Chi-square (χ^2) test was used for dichotomic variables. Odds ratio (OR) with 95% confidence interval (CI) was evaluated. Correlations between variables were evaluated by Pearson (r) or Spearman (r_s) correlation. Receiver operator characteristic (ROC) analysis was performed to determine sensitivity and specificity of a cut-off value for vitamin D in detecting G2 and metastasized tumors. PFS was defined as the time interval between diagnosis and disease progression. DSS has been calculated from the date of GEP-NET diagnosis to patient disease-related death or the end of data collection. Kaplan–Meier analysis and log-rank test were used to evaluate both PFS and DSS. Cox proportional hazard regression model was used to perform univariate and multivariate regression analysis.

Statistical analysis was made using SPSS Software (PASW Version 21.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (version 5.0, La Jolla, CA, USA). ROC analysis was made using MedCalc Software (version 12.3.0, Mariakerke, Belgium). A p value < 0.05 was considered statistically significant.

Results

Study population

We retrospectively evaluated 133 patients affected by well-differentiated, low-grade GEP-NET. Patients who did not meet the inclusion criteria or did not complete the baseline assessment were excluded from the study (Fig. 1). A final number of 75 GEP-NETs were included in this study and were matched with 123 healthy controls coming from the same geographical area. The clinical characteristics of both groups were summarized in Table 1.

Among GEP-NETs, 64 cases (37 males and 27 females) had a sporadic GEP-NET and 11 (3 males and 11 females) had MEN1 without PHPT. Thirty-seven (49.3%) patients had pancreatic NET, 17 (22.7%) had a gastro-duodenal NET, 14 (18.7%) had a NET of the ileum and 7 (9.3%) had a tumor in the appendix or rectum. Most patients (88%) had

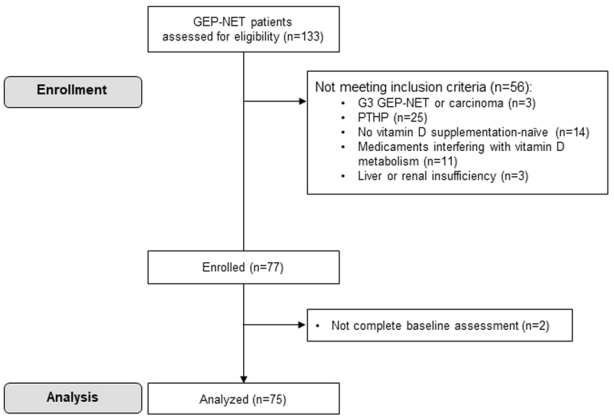

Fig. 1 Flow diagram of the included patients. Abbreviation: G tumour grade, GEP-NET gastroenteropancreatic neuroendocrine tumors, PHPT primary hyperparathyroidism

non-functioning GEP-NET. Among functioning GEP-NETs ($n = 9$), 8 were pancreatic NETs (5 insulinomas, 2 gastrinomas, and 1 VIPoma) and one was a NET of the ileum associated with carcinoid syndrome. At the time of the evaluation, 44 (58.6%) patients had undergone endoscopic ($n = 5$) or surgical resection ($n = 39$) of primary tumor, among which 15 (20%) cases underwent a surgical resection of duodenum ($n = 7$) or ileum ($n = 8$) and 24 (32%) underwent other types of abdominal surgery, including distal pancreatectomy, appendicectomy or hemicolectomy. Particularly, due to the low number of patients who underwent the duodenal or ileal resection, we considered these patients as a single group named as "small bowel resection" group. Among patients who underwent surgery for a pancreatic NET ($n = 20$, both partial and total pancreatectomy), 8 assumed pancreatic enzymes replacement therapy.

Thirty-five (46.7%) GEP-NETs were treated with SSAs at the time of evaluation, including 24 (32%) cases treated for a period longer than ≥18 months (long-term) [14], and 11 (14.7%) cases treated for less than 18 months (short-term). Median time of treatment was 44.5 months (range 18–163) in the long-term treated group and 5.0 months (range 1–11) in the short-term group ($p < 0.0001$). At the time of the evaluation, 35 (46.7%) patients were tumor-free, 17 (22.7%) had a stable disease and 23 (30.7%) had a progressive disease.

Compared to controls, GEP-NET patients had a slightly, but not significant, higher prevalence of obesity (33.3% vs 19.5% of cases) and a significantly larger WC ($p = 0.01$, Table 1). A significant direct correlation was observed between BMI and WC in both groups ($r = 0.9$, $p < 0.001$, in GEP-NETs and $r = 0.29$, $p = 0.009$, in controls). Moreover, in GEP-NET patients, BMI and WC moderately correlated with tumor size ($r_s = 0.33$, $p = 0.005$, and $r_s = 0.32$, $p = 0.008$, respectively), and with Ki67 index ($r_s = 0.28$, $p = 0.019$, and $r_s = 0.36$, $p = 0.004$, respectively). A strong correlation between Ki67 index and tumor size was observed ($r_s = 0.44$, $p < 0.001$).

Table 1 Clinical characteristics of GEP-NET patients and healthy controls matched by sex, age, and BMI

Parameter	GEP-NETs ($n = 75$)	Controls ($n = 123$)	p value	χ^2
Sex				
F	35 (46.7%)	66 (53.7%)	0.34	0.91
M	40 (53.3%)	57 (46.3%)		
Age years	55.9 ± 14.2	54.7 ± 15.0	0.54	n.a.
BMI kg/m^2	27.7 ± 5.4	26.6 ± 3.4	0.11	n.a.
BMI categories:				
Underweight	2 (2.7%)	2 (1.6%)	0.15	5.3
Normal weight	19 (25.3%)	40 (32.5%)		
Overweight	29 (38.7%)	57 (46.4%)		
Obesity	25 (33.3%)	24 (19.5%)		
Waist circumference - cm	93.3 ± 15.5	87.9 ± 10.7	**0.01**	–
25(OH)D levels ng/mL	17.9 ± 7.8	24.2 ± 7.7	**<0.0001**	–
Vitamin D categories:				
Severe deficiency	13 (17.3%)	2 (1.6%)	**<0.001**	25.2
Deficiency	30 (40.0%)	34 (27.6%)		
Insufficiency	26 (34.7%)	59 (48.0%)		
Sufficiency	6 (8.0%)	28 (22.8%)		
Albumin-corrected serum Ca mg/dL	9.3 ± 0.6	9.3 ± 0.4	0.69	–
Phosphorus mg/dL	3.4 ± 0.6	3.9 ± 0.4	**<0.0001**	–
PTH pg/mL	56.3 ± 31.7	26.2 ± 15.9	**<0.0001**	–
Tumor grading:				
G1	41 (54.7%)	–	–	–
G2	34 (45.3%)			
Tumor stage at diagnosis:				
Localized disease	54 (72.0%)	–	–	–
Metastasis	21 (28.0%)			

Continuous variables are reported as mean with standard deviation, whereas categorical variables are reported as numbers (percentages). Statistical analysis was performed by *t*-test or Man–Whitney test or chi-square test (χ^2), as appropriate. A p value in bold type indicates a significant difference ($p < 0.05$). Abbreviation: *BMI*, body mass index; 25(OH)D, 25-hydroxyvitamin D; *Ca*, calcium; *G*, grade; *GEP-NETs*, gastroenteropancreatic neuroendocrine tumors; *PTH*, parathyroid hormone.

Mean serum albumin-corrected calcium (Ca) and phosphorus levels were within the normal range in both groups, although GEP-NETs had significantly lower phosphorus concentration than controls ($p < 0.0001$; Table 1 and Fig. 2A, B). GEP-NET patients showed significantly higher mean PTH levels than controls (56.3 ± 31.7 vs 26.2 ± 15.9 pg/mL, $p < 0.0001$; Fig. 2C) but within the normal range, except for 4 patients with sporadic GEP-NET who presented PTH concentration above the upper limit. A

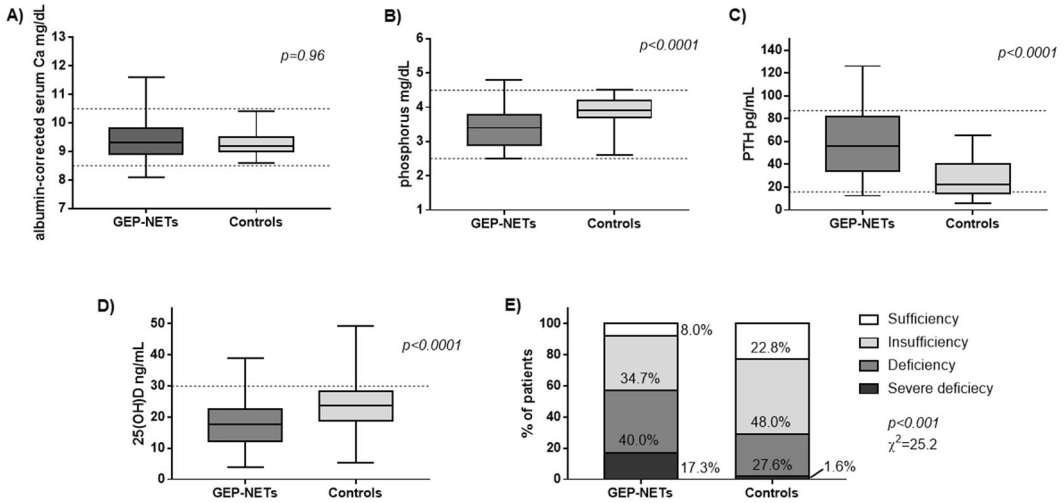

Fig. 2 Calcium, phosphorus, PTH and vitamin D levels in GEP-NETs ($n = 75$) and healthy matched controls ($n = 123$). Difference in albumin-corrected serum calcium (**A**), phosphorus (**B**), parathyroid hormone (**C**) and vitamin D in GEP-NET patients compared to controls. The dashed lines indicate the lower and the upper value of the normal range (**A**–**C**) and the lower limit of vitamin D sufficiency (**D**). **E** Vitamin D status according to the Endocrine Society guidelines [34] in the entire cohort of GEP-NETs in comparison to controls. Statistical analysis was performed by t test or Mann-Whitney test and Chi-square test, as appropriate. Conversion Factors between 'Conventional' and the International System (SI) Units: calcium: 1 mg/dl = 0.25 mmol/L; PTH: 1 pg/mL = 0.1 pmol/L; 25(OH)D: 1 ng/mL = 2.5 nmol/L. Abbreviation: 25(OH)D 25-hydroxyvitamin D, Ca calcium, GEP-NET gastroenteropancreatic neuroendocrine tumor, PTH parathyroid hormone

strong negative correlation was found between phosphorus and PTH levels in controls ($r_s = -0.49$, $p < 0.001$), whereas only a trend was observed in GEP-NET patients ($r_s = -0.49$, $p = 0.056$). No significant correlation was found between PTH and serum albumin-corrected Ca in both group ($r = -0.49$, $p = 0.82$, in GEP-NETs and $r_s = -0.17$, $p = 0.29$, in controls).

Serum 25(OH)D levels

Mean 25(OH)D levels were significantly lower in GEP-NETs compared to control group (17.9 ± 7.8 vs 24.2 ± 7.7 ng/mL, $p < 0.0001$; Fig. 2D). Particularly, vitamin D deficiency and severe deficiency was observed in 57.3% ($n = 20$) and 17.3% ($n = 13$) of GEP-NETs, compared to 24.6% and 1.6% in controls, respectively ($\chi^2 = 25.2$, $p < 0.001$; Table 1 and Fig. 2E). Only 6 (8%) GEP-NETs had vitamin D sufficiency compared to 28 (22.8%) control cases (Fig. 2E).

25(OH)D levels did not correlate with BMI ($r = -0.03$, $p = 0.79$) and WC ($r = -0.11$, $p = 0.39$) in GEP-NETs, whereas 25(OH)D significantly correlated with both parameters in the control group ($r = -0.45$, $p < 0.001$ and $r_s = -0.28$, $p = 0.01$ for BMI and WC, respectively). Moreover, in the control group, 25(OH)D levels significantly correlated with albumin-corrected Ca ($r = -0.46$, $p = 0.003$). No significant correlations were found between 25(OH)D levels and age ($r = -0.22$, $p = 0.06$ and $r = 0.01$, $p = 0.89$ in GEP-NETs and controls, respectively), sex ($r_s = 0.14$, $p = 0.23$ and $r_s = 0.14$, $p = 0.11$ in GEP-NETs and controls, respectively), and PTH ($r = 0.15$, $p = 0.45$ and $r_s = 0.20$, $p = 0.22$ in GEP-NETs and controls, respectively).

Considering the site of the primary tumor, patients with NET of the appendix and rectum showed a trend toward higher 25(OH)D levels (mean 23.5 ± 9.8 ng/mL) compared to pancreatic NET (mean 18.9 ± 7.1 ng/mL), gastro-duodenal NET (mean 6.6 ± 7.2 ng/mL) and those with NET of the ileum, which showed the lowest 25(OH)D levels (mean 13.6 ± 8.3 ng/mL; $p = 0.049$, Fig. 3A). In details, severe vitamin D deficiency was observed in 42.9% of patients with NET of the ileum had severe vitamin D deficiency, in 23.5% of those with gastro-duodenal NET, in 14.3% of those with ileal NET, and in 5.4% of those with pancreatic NET (Fig. 3B). To note, no cases within the group of ileal NET achieved the vitamin D sufficiency.

Mean 25(OH)D levels were significantly lower in patients who underwent small bowel resection compared to those who underwent other types of abdominal surgery (12.5 ± 5.5 vs 20.8 ± 6.9 ng/mL, $p = 0.007$; Fig. 3C), but no differences were observed in comparison to those who underwent an endoscopic tumor resection (mean 18.9 ± 9.9 ng/mL) or to those who did not undergo to surgery (mean 17.8 ± 8.3 ng/mL; Fig. 3C). Particularly, none of the patients who underwent small bowel resection had sufficient vitamin D levels (Fig. 3D). These patients had a significant increased risk of severe vitamin D deficiency compared to all other groups (OR = 3.18, 95% CI = 1.37–3.38, $\chi^2 = 6.7$, $p = 0.01$). Moreover, within the small bowel resection

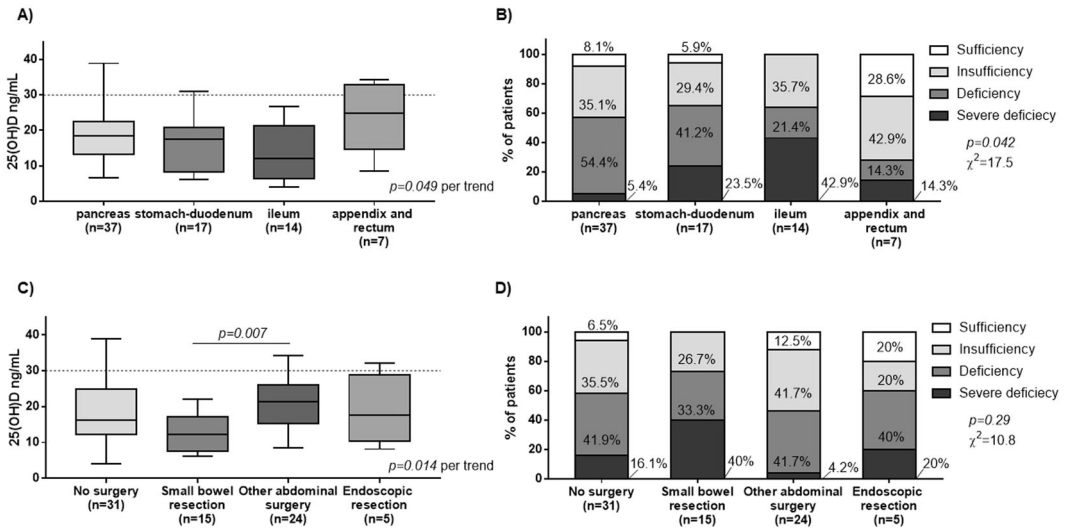

Fig. 3 Vitamin D levels according to primary tumor site and type of surgery. 25(OH)D levels (**A**) and vitamin D status evaluated according to the Endocrine Society guidelines [34] (**B**) among the different primary tumor site, showed that patients with ileal NET had lower vitamin D levels compared to the other primary tumor sites. 25(OH)D levels (**C**) and vitamin D status according to the Endocrine Society guidelines [34] (**D**) considering the type of surgery performed for the primary tumor resection. Patients who underwent small bowel resection had lower 25(OH)D levels than those who underwent other types of abdominal surgery. The dashed lines indicate the lower limit of vitamin D sufficiency. Statistical analysis was performed with the Kruskal–Wallis test followed by Dunn's post-hoc test and Chi-square test. Conversion Factors between 'Conventional' and the International System (SI) Units: 25(OH)D: 1 ng/mL = 2.5 nmol/L. Abbreviation: 25(OH)D 25-hydroxyvitamin D, GEP-NET gastroenteropancreatic neuroendocrine tumor

group, no significant difference in 25(OH)D concentration was observed between patients with duodenal or ileal resection ($p > 0.99$).

Among patients who underwent pancreatic surgery, 25 (OH)D levels were not different between those having pancreatic enzymes replacement therapy and those without (mean 17.7 ± 6.2 vs 17.1 ± 6.2 ng/mL, $p = 0.79$). No significant difference in 25(OH)D levels was observed in patients with SSA-therapy naive compared to those currently treated with SSAs (mean 18.1 ± 6.9 vs 17.7 ± 8.9 ng/mL; $p = 0.78$), although those treated with SSAs had a slight increased rate of vitamin D severe deficiency (22.9% vs 12.5%, respectively). Considering the long-term (≥18 months) and the short-term (<18 months) SSA treatment, no difference in mean 25(OH)D levels was observed (18.9 ± 9.6 vs 15.1 ± 71 ng/mL, respectively; $p = 0.25$).

Vitamin D and parameters of tumor aggressiveness

Mean 25(OH)D levels were significantly lower in patients with G2 tumors compared to those with G1 (15.6 ± 7.8 vs 19.9 ± 7.4 ng/mL, $p = 0.016$). Particularly, 26.5% and 47.1% of G2 patients had vitamin D severe deficiency or deficiency, compared to 9.8% and 34.1% of G1 patients, respectively ($\chi^2 = 9.02$, $p = 0.029$; Fig. 4A).

At the time of the patient evaluation, patients with progressive disease had significantly lower 25(OH)D levels compared to those who were tumor free after surgery (mean 12.6 ± 5.7 vs 19.6 ± 7.3 ng/mL, $p = 0.002$) and to those with stable disease (mean 21.5 ± 8.2 ng/mL, $p = 0.001$). Particularly, none of the patients with progressive disease had sufficient levels of vitamin D, whereas vitamin D severe deficiency and deficiency was observed in 34.8% and 48.8% of cases, respectively (Fig. 4B). On the contrary, severe vitamin D deficiency was observed only in 11.4% and 5.9% of patients with tumor free or stable disease, respectively ($\chi^2 = 13.2$, p = 0.04; Fig. 4B).

The ROC analysis showed that a cut-off of 25(OH)D ≤ 13.5 ng/mL could be associated with G2 tumors with a specificity of 86.5% but with a low sensitivity of 48.5% ($p = 0.012$, Fig. 4C), whereas a cut-off of 25(OH)D levels ≤16.3 ng/mL could be associated with tumor progression with a sensitivity of 80.9% and specificity of 69.4% ($p < 0.001$, Fig. 4D).

Vitamin D and clinical outcome

Median progression-free survival (PFS) of the entire cohort was 65 (range 1–228) months. Median PFS for patients with vitamin D deficiency was 84 months, whereas it was not reached in the other groups. Patients with vitamin D deficiency and insufficiency had a significantly shorter PFS compared to those with sufficient vitamin D concentration (HR = 3.44, 95% CI = 1.11–10.63 and HR = 3.47, 95% CI = 0.52–22.93, respectively, $p = 0.014$; Fig. 5A). Moreover, PFS was significantly shorter in patients with vitamin D deficiency compared to those with vitamin D sufficiency (HR = 2.27, 95%CI 1.08–4.75, $p = 0.03$). At univariate

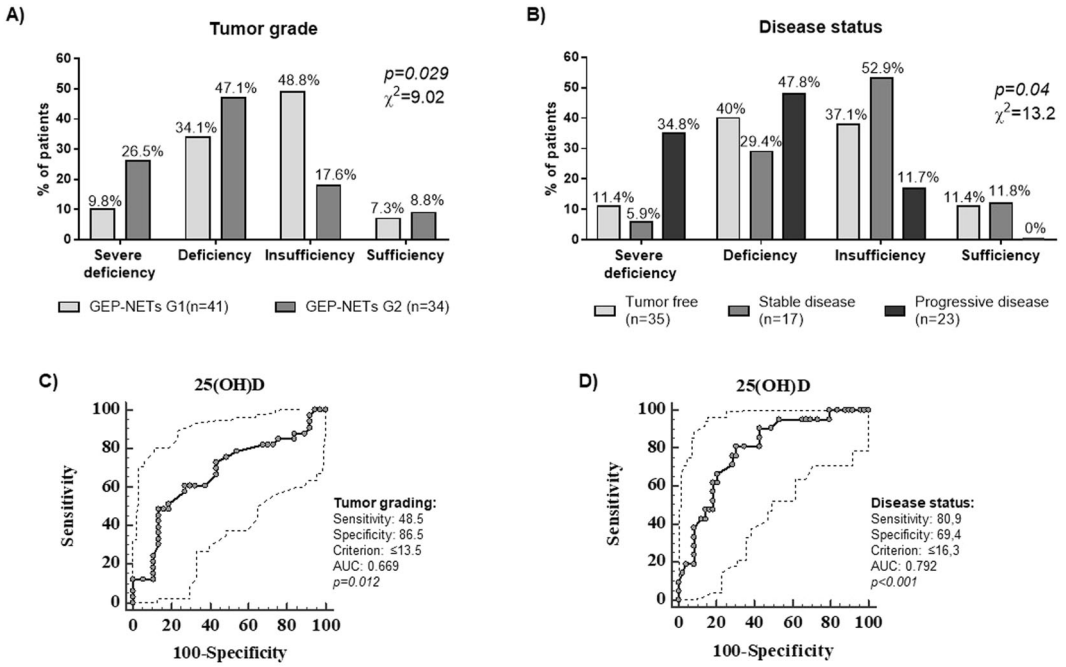

Fig. 4 Vitamin D status and parameters of tumor aggressiveness. Evaluation of vitamin D status according to the Endocrine Society guidelines [34] according to tumor grade (**A**) and disease status (**B**). ROC curve showed that a cut-off of 25(OH)D ≤ 13.5 and ≤16.3 ng/mL could be associate with G2 tumors (**C**) and progressive disease (**D**), respectively. Statistical analysis was performed by Chi-square test and Receiver operator characteristic (ROC) curve analysis

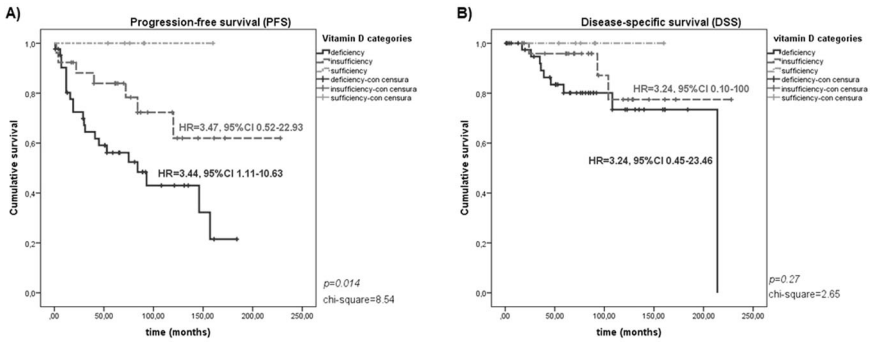

Fig. 5 Progression-free survival and disease-specific survival according to the vitamin D status. **A** Patients with vitamin D insufficiency and deficiency had a significantly shorter progression-free survival (PFS) compared to patients with vitamin D sufficiency. **B** Vitamin D status did not significantly affect disease-specific survival (DSS); however, patients with vitamin D insufficiency and deficiency had a higher risk of a shorter DSS. Statistical analysis was performed by Kaplan–Meier curve and log-rank test

analysis, vitamin D, tumor grade and stage, and sex significantly correlated with PFS (Table 2A). However, at multivariate analysis only tumor grade and stage were confirmed to be associated with PFS (Table 2A).

Median disease-specific survival (DSS) of the entire cohort was 77 months (range 1–228). No correlation was observed between 25(OH)D levels and DSS (Fig. 5B and Table 2B). However, patients with vitamin D insufficiency or deficiency presented a higher, but not significant, risk of shorter DSS (Fig. 5B). At univariate and multivariate analysis, only tumor grade and stage were significantly associated with DSS (Table 2B).

Discussion

Vitamin D deficiency and insufficiency is a worldwide health problem that afflicts more than one billion children and adults [35]. Since vitamin D showed pleiotropic effects in different system and organs [1–5, 36, 37], the consequences of vitamin D deficiency cannot be underestimated [35]. Recent evidence shows that sufficient 25(OH)D levels are associated with a reduced cancer risk and mortality [6, 7]. The role of vitamin D in patients with NET arises only in the last decade [13]. Two in vitro studies demonstrated that 1α,25-dihydroxyvitamin D3 and its

Table 2 Univariate and multivariate analysis correlating clinicopathological parameters with progression-free survival (A) and disease-specific survival (B)

Parameters	Univariate			Multivariate		
	p	HR	95% CI	*p*	HR	95% CI
(A) Progression-free survival						
Vitamin D	**0.006**	0.34	0.15–0.73	0.46	0.73	0.36–1.69
Tumor grade	**<0.001**	5.07	2.23–11.51	**0.03**	2.80	1.09–7.18
Tumor stage	**<0.001**	11.51	4.93–26.85	**0.001**	5.35	1.96–14.60
Sex	**0.04**	0.46	0.21–0.96	0.44	0.70	0.29–1.71
BMI	0.20	1.37	0.84–2.22	–	–	–
(B) Disease-specific survival						
Vitamin D	0.12	0.38	0.11–1.28	–	–	–
Tumor grade	**0.006**	17.32	2.23–134.43	**0.025**	11.03	1.36–89.62
Tumor stage	**0.001**	8.48	2.42–29.73	**0.027**	4.45	1.19–16.67
Sex	0.77	0.84	0.25–2.75	–	–	–
BMI	0.47	1.31	0.63–2.76	–	–	–

Vitamin D was considered according to the Endocrine Society Guidelines [32] in three categories: sufficiency, insufficiency, and deficiency. Tumor grade was considered as G2 vs G1. Tumor stage at diagnosis was considered as the presence of metastasis vs localized diseased. Sex was considered as female vs male. BMI was considered in three categories: underweight + normal weight, overweight, and obesity. Statistical analysis was performed by Cox proportional hazard regression; only those parameters that were statistically significant at the univariate analysis were included in the multivariate model. A *p* value in bold type indicates a significant difference ($p < 0.05$). Abbreviation: *95%CI* confidence interval; *HR* hazard ratio.

analogs inhibit cell growth and metastatic potential processes in rat insulinoma cell line expressing vitamin D receptor [38, 39]. Only few studies investigated vitamin D levels in NEN patients, showing a high frequency of vitamin D deficiency [14–17]. However, the majority of these studies included small cohorts NET patients [14, 15, 21] or sometimes reported contrasting results [14–17, 21].

In this study, we investigated a selected cohort of patients affected by low-grade GEP-NET. As showed by Massironi et al. [16], we confirmed that vitamin D deficiency and severe deficiency is highly prevalent among GEP-NET patients compared to healthy controls (57.3% vs 29.2%), whereas only a small percentage of patients reported sufficient vitamin D levels (8%). In our GEP-NET cohort, 42.7% of patients reported vitamin D insufficiency/sufficiency, which is in line to what previously reported in the literature where 25(OH)D levels >20 ng/mL were found in a mean of 46.7% of cases (ranging from 31.4% to 68.5% according to the different studies) [14–17].

Importantly, in our study, healthy controls were matched not only for age and sex, as in the study by Massironi et al. [16], but were matched also for BMI and were coming from the same geographic area, to reduce potential bias in the evaluation of vitamin D levels. 25(OH)D levels inversely correlate with BMI only in controls, confirming what already reported by Robbins et al. [17], underlying that other factors may affect vitamin D levels in GEP-NET patients. Although there was no difference in BMI, GEP-NET patients showed a larger WC in comparison to controls, probably related to a different type of dietary pattern and a higher prevalence of metabolic syndrome in GEP-NETs [27, 29]. This result is not surprising since it is well demonstrated that for any given BMI, the variation in WC is considerable [40]. Moreover, different to the BMI, the WC is a measurement of the visceral obesity [40] and correlates with metabolic syndrome [41]. The high prevalence of overweight and obesity in our cohort of patients could be linked to the lack of patients with high-grade tumor (G3). In fact, according to Maasberg et al. [42] patients with G3 disease had a significantly higher prevalence of malnutrition than those with G1 or G2 tumor. To note, we found a correlation between BMI and WC with Ki67% and tumor size, which might reflect a correlation between metabolic dysfunction and impaired insulin status with tumor size and invasiveness, as demonstrated in other solid tumors [43–45].

Evaluating the other parameters associated to vitamin D metabolism, we found higher PTH levels in GEP-NETs compared to controls. It is important to underline that patients with PHPT associated to MEN1 were excluded from this study, therefore the high PTH levels likely represent a condition of secondary hyperparathyroidism due to hypovitaminosis D. The lower serum phosphorus levels found in GEP-NETs could be caused by hypovitaminosis D and high PTH levels [46]. To note, our center is specialized in MEN1 patients, explaining why in the initial screened cohort included a higher number of patients with MEN1 and PHTP, which were then excluded from the study. For the same reason, we had in the final cohort a higher number of patients with pancreatic NET compared to other tumor localizations.

In this study, we analyzed different parameters that could be associated with hypovitaminosis D in GEP-NET patients [13]. For the first time, we demonstrated that 25(OH)D levels correlated with primary tumor site among GEP-NETs. Particularly, we showed that patients with ileal NET had a trend to lower 25(OH)D levels compared to the other types of GEP-NETs, followed by patients with gastric-duodenal NET, whereas only a small percentage of patients with NET of the appendix and rectum had vitamin D deficiency. Although these results could appear in contrast to what previously reported, it is important to note that in the study by Motiylewska et al. the authors analyzed the GEP-NET patients as one group and compare them to patients with NETs of the lung or other types of NETs [21], whereas in the study by Massironi et al. patients with pancreatic NET were compared to all other type of gastrointestinal NET [16]. Here, for the first time, we analyzed the primary tumor site of GEP-NETs separately, evaluating pancreatic vs gastric-duodenal vs ileal vs appendix and rectum NETs. The lower 25(OH)D levels found in patients with ileal NET could be correlated to the type of primary tumor resection, which could modify the gastrointestinal tract [13]. Opposite to Fiebrich et al. [14], Robbins et al. [17] reported that abdominal surgery had an impact on vitamin D levels. In our study, we found that patients who underwent small bowel resection presented lower 25(OH)D levels than those who underwent other types of abdominal surgery and had a higher rate of vitamin D severe deficiency. This is in accordance with evidence from the literature showing that the resection of the ileum, especially of the terminal tract [47, 48]. Vitamin D is a lipid-soluble vitamin that depend on bile salts for normal absorption. The resection of the ileum leads to insufficient intra-intestinal bile salt concentrations, which might lead to fat-soluble vitamins malabsorption, including vitamin D [47, 48]. It is important to underline that in our study we evaluated patients who underwent to ileum or duodenum resection as a single group ("small bowel resection") because of the low number of patients within each group. However, although the resection of the duodenum differs significantly from resection of the ileum in the way absorption of nutrients [49], increasing evidence showed that also the duodenum could be involved in the absorption of vitamin D. In the duodenum, vitamin D is released from the food and emulsified into lipid droplets thanks to the presence of a digestive enzyme (such as the cholesterol ester hydrolase, the lipase and the pancreatic lipase-related protein) and enzymes present at the brush-border membrane level of the epithelial cells [50, 51]. This step is important because only the free forms of fat-soluble vitamins are thought to be absorbed by the intestinal cell [50]. Moreover, two different transporters, the Scavenger Receptor Class B type I and the CD36, which facilitate the intestinal uptake of lipid micronutrient, including the vitamin D, are expressed at the brush-border membrane of the duodenum as well as of the other intestinal tracts [52, 53].

SSAs play a central role in the treatment of patients with well-differentiated GEP-NET, but they may cause transient or permanent gastrointestinal adverse events, including the suppression of pancreatic exocrine secretion, the impairment of hepatic bile acid and the suppression of various gut hormones [54]. The consequent diarrhea and steatorrhoea may have an impact on fat-soluble vitamins absorption, including vitamin D. In accordance with previous studies [17, 21], but in contrast to Massironi et al. [16], we demonstrated that SSAs treatment did not affect significantly the 25(OH)D levels, although patients treated with SSAs presented a slightly increased rate of severe vitamin D deficiency. Moreover, we also compared short-term (≤18 months) vs long-term SSA treatment, without finding differences in 25(OH)D levels. This result was similar to Fiebrich et al. [14], which reported that the duration of SSAs therapy did not influence vitamin D levels in long-term treated cases.

Pancreatic enzyme replacement treatment is suggested in cases presenting diarrhea and steatorrhea, which are frequent in GEP-NETs [55]. Pancreatic enzymes supplementation should improve fat uptake and fat-soluble vitamins absorption. In our cohort, eight patients were supplemented with pancreatic enzymes after pancreatic resection, but they did not show difference in 25(OH)D levels compared to patients who were not treated. However, due to the retrospective nature of the study, data on diarrhea/steatorrhea were lacking, as well as the number of patients treated with pancreatic enzyme were too low, raising up a potential bias in the interpretation of the result.

Differently from Massironi et al. [16], but in line with studies in other types of solid tumors [56, 57], we demonstrated that vitamin D deficiency was associated with higher grade (G2) tumor and disease progression. This discrepancy could be due to the relative limited number of patients included in both studies due to the rare incidence of GEP-NETs in the general population. We also found that a cut-off of 25(OH)D levels ≤16.3 ng/mL could be associated to an increased risk of tumor progression.

At univariate analysis, patients with vitamin D deficiency and insufficiency had a shorter PFS compared to those with sufficient 25(OH)D levels. However, this result was not confirmed at the multivariate analysis including tumor grade and stage. It is important to underline that the low number of patients with sufficient 25(OH)D levels ($n = 6$) represents a strong limitation for the interpretation of the result. Therefore, it results difficult to draw a definitive conclusion. Massironi et al. [16] also showed an inverse correlation between vitamin D and overall survival. Unlike this study, we did not find a significant correlation between vitamin

status and DSS, although patients with vitamin D deficiency and insufficiency showed a trend to a higher risk of shorter DSS. To note, in our study we evaluated the DSS and not the overall survival, reducing the number of specific events observed in our cohort ($n = 13$). In contrast, Robbins et al. [17] did not reported a correlation between vitamin D and clinical outcome, but this result is probably due to the short time of follow-up (2 years). In our cohort, we were able to confirm that tumor grade and stage are two strong prognostic factors of tumor progression and patient survival [58]. Massironi et al. [16] also demonstrated that the overall survived was improved in patients who received vitamin D supplementation. Although we did not investigate the impact of vitamin D supplementation on disease progression, 55 (73.3%) patients of our cohort were than supplemented with Vitamin D. It has been demonstrated that only 50–55% of GEP-NET patient reach sufficient vitamin D levels after supplementation or intensive dietician input [17, 59], supporting the hypothesis that other factors in GEP-NET patients (including the type of underwent surgery) could changes the 25(OH)D concentration.

Limitations of this study warrant some considerations. First, its retrospective and cross-sectional nature did not allow to clearly identify 25(OH)D levels as a prognostic factor of GEP-NET aggressiveness, and the suggested cut-off value should be viewed with caution until validated in prospective trials. Second, the number of included patients was relatively low, particularly when we divided our cohort in different subgroups (i.e. according to tumor site or type of surgery). Although we included a higher number of patients and controls than those indicated by the power analysis, the interpretation of the results should be taken with prudence. In fact, the power analysis was retrospectively conducted with the aim of determine the power of the study and not to calculate a priori the sample size. Therefore, our results need to be further validated in prospective studies including a larger cohort of patients. However, considering the low incidence of GEP-NETs, we were able to include a very well-characterized and homogeneous cohort of patients, including only those patients with well-differentiated and low-aggressive tumors. Differently from the previous studies, we excluded all GEP-NET patients with HPTH or with other criteria that are known to interfere with vitamin D metabolism. Importantly we evaluated also other parameters involved in the vitamin D metabolism, including serum Ca, phosphorus and PTH. Finally, the single-center study allowed to increase the homogeneity of the two studied groups, since we included participants living in the same geographical area, with the same effect of latitude on vitamin D levels. All these factors have allowed us to gain a better understanding of vitamin D status in GEP-NET patients.

In conclusion, the monitoring of 25(OH)D levels is relevant in GEP-NETs because of the highly prevalence of vitamin D deficiency that could be associated with high tumor grade and disease progression. Therefore, vitamin D supplementation should be considered in all patients with vitamin D deficiency or insufficiency because it could have a positive impact on patient prognosis.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Naples "Federico II" Medical School (n. 201/17).

Consent to participate Informed consent was obtained from all subjects involved in the study.

Consent for publication All authors read and approve the final version of the manuscript.

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