SAPIENZA
UNIVERSITÀ DI ROMA

DOTTORATO DI RICERCA IN SCIENZE ENDOCRINOLOGICHE

XXXV CICLO

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TESI DI DOTTORATO

REDISCOVERING BIOMARKERS IN FOR THE DIAGNOSIS AND EARLY TREATMENT RESPONSE IN NEN
REBORN STUDY

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ABSTRACT

Introduction: Neuroendocrine neoplasms (NENs) are heterogeneous in terms of primary site, behavior, and response to treatment. The possibility to rely on diagnostic and prognostic circulating biomarkers is an unmet need in NENs. Despite promising, the clinical role of circulating angiogenic markers remains unclear. In addition, liquid biopsy is currently receiving growing attention in oncology, but data in NENs are available only for circulating nucleic acids and tumor cells while the potential role of circRNA sequencing from tumor educated platelets (TEPs) has never been explored. The aim of this study was to evaluate the role of angiogenic markers and circRNA sequencing from TEPs in NENs diagnosis and prognosis.

Materials and Methods: We performed a prospective observational study including 46 consecutive patients with proven NENs of pulmonary and gastro-entero-pancreatic (GEP) origin and 29 controls. Circulating pro-angiogenic factors were measured by ELISA assay, and ANG2 tissue expression was evaluated by immunohistochemistry. A limited subgroup of patients, affected by well-differentiated GEP NET, grade G1 or G2, naïve to any medical treatment, was also included in a proof-of-concept pilot study, for analyzing the expression profile of circRNA derived from TEPs, both at baseline and early follow-up (after 3 months of treatment).

Results: The study demonstrated a significantly higher level of ANG2, ANG1, sTIE2, and PROK2 in patients affected by NENs compared to controls. In the subgroup of patients with NENs, ANG2 levels were higher in poorly differentiated NENs (4.9, 2.8–7.4) than in well-differentiated (3.2, 1.7–6.4) ng/ml, p = 0.046 and in tumor stage 3–4 compared to stage 1–2: 4.2 (2.7–8.7) vs 2.7 (1.5–5.7), p = 0.044. ANG2 and PROK2 were significantly higher in patents with progressive disease compared to stable disease at the moment of sampling: ANG2 = 6.3 (4.0–11.0) vs 2.7 (1.7–4.7) pg/ml, p = 0.001; PROK2 = 29.2 (28.4–32.3) vs 28.4 (28.1–28.9) pg/ml, p = 0.035. ANG2 was also higher in patients who developed progression (or died) during the follow-up (one year after the enrollment) than in patients with stable disease (2.3 (1.5-3.8) ng/ml *vs* 6.3 (4.2-10.1) ng/ml, p<0.001). Immunohistochemistry confirmed ANG2

and PROK2 expression in tumor specimens. We identified a large number of circRNA in this study (98,735), of which 63,562 were not previously annotated and 35,173 annotated. To investigate the potential role of circRNAs expression profile from TEPs as diagnostic and prognostic biomarkers, a bioinformatic analysis is ongoing to evaluate differently expressed circRNA from TEPs between patients and controls and in the same patients before and after treatment.

Conclusions: We demonstrated higher levels of angiogenic markers in NENs, with a correlation between ANG2 serum levels and NENs morphology and staging. In both GEP and lung NENs, ANG2 and PROK2 are higher in case of tumor progression, suggesting a potential role as prognostic markers in NENs patients. The study also demonstrated that TEPs are a good source of circRNA in patients affected by NENs. The bioinformatics analyses are currently ongoing and could be the base for the development of novel markers for the diagnosis and follow-up of patients affected by NENs.

SUMMARY

1. INTRODUCTION

1.1 Neuroendocrine neoplasms

Definition and epidemiology

Neuroendocrine Neoplasms (NENs) are a group of neoplasms that arise from neuroendocrine cells throughout the body, characterized by the ability to produce hormones and peptides[1]. The term NENs included the well-differentiated forms, also known as Neuroendocrine Tumors (NETs), and the poorly differentiated neoplasms, also known as Neuroendocrine Carcinomas (NECs)[2].

NENs are heterogeneous in terms of localization since they can appear in every part of the body. The most frequent localization is the gastro-entero-pancreatic (GEP) followed by bronchopulmonary systems, but NENs can arise also in the thyroid, pituitary, adrenal glands, skin, breast, ovaries, and prostate[3]. According to SEER (Surveillance, Epidemiology, and End Results Program) data, the most frequent sites of primary tumors in females are the lung, stomach, appendix, and cecum, while in males the are thymus, duodenum, pancreas, jejunum/ileum, and rectum[4]. Neuroendocrine neoplasms are more frequent in males and their incidence increases with age[5].

The annual incidence of NEN is increasing in the last 30 years, even if the reasons underlying this rise have not been completely identified. The improvement of the diagnostic techniques certainly plays a role[6]. From the analysis of SEER data, the incidence of NEN in the USA, in 2004, was 5.25 cases per 100,000 inhabitants, with a significant increase over time[4]. Subsequent analysis revealed that the incidence rises from 1.09 per 100,000 in 1973 to 6.98 per 100,000 inhabitants in 2012 (a 6.4-fold increase), for all sites, stages, and grades[3]. In a population-based retrospective cohort study in Canada, from 1994 to 2009, the incidence increased from 2.48 to 5.86 cases per 100,000 inhabitants per year (2.36-fold)[7]. European data can be derived from a large database provided by the RARECARE project, "surveillance of rare cancers in Europe", which included patients diagnosed with cancer from 1978 to 2002. The overall incidence rate for all NETs excluding lung was 2.5 per 100,000 inhabitants, rising

to 4 per 100,000 inhabitants in patients older than 65 years[5]. The incidence of NEN in Italy, according to the AIRTUM register, is 4.15 cases per 100,000 per year (2.697 cases per year)[8].

The prevalence of NEN is also increasing over time. Estimating the 20-year limited-duration prevalence using SEER data, Dasari *et al*. demonstrated a substantial increase, from 0.006% in 1993 to 0.048% in 2012[3]. This rise can be explained by the increased incidence and by the availability of new treatments and the proportion of NENs with indolent behavior, which increase the survival of the patients. However, also other factors could have played a role. The risk factors for neuroendocrine neoplasms are not fully understood, and the results of the available studies are sometimes contrasting. Two meta-analyses have been conducted to draw conclusions on this topic[9,10]. The first one demonstrated that first-degree family history of neoplasms and diabetes mellitus are risk factors for pancreatic NENs[9]. The second meta-analysis included studies on all GEP-NENs and confirmed the role of family history in NENs development. Interestingly, some risk factors can vary according to primary sites. Diabetes mellitus, obesity, and smoking are risk factors for pancreatic NENs, while only smoking is a risk factor for small intestine NENs[10]. Our group recently took part in an Italian three-centric case-control study that confirmed that a family history of non-neuroendocrine gastro-intestinal cancer, type 2 diabetes mellitus, and obesity were all risk factors for GEP-NENs. Diabetes mellitus was also associated with more advanced and progressive diseases. Stratifying risk factors for the primary site, the study demonstrated the role of diabetes mellitus and obesity in the pancreatic NENs while a family history of non-neuroendocrine GEP cancer and obesity had a role in small intestine NENs[11].

Clinical presentation

NENs are heterogeneous also regarding the clinical presentation. In some cases, NENs can be functioning, with an overproduction of hormones that causes the typical clinical syndromes associated with NENs[12].

Carcinoid syndrome is usually due to small intestinal NENs with liver metastasis. Classical carcinoid syndrome is characterized by diarrhea, abdominal pain, and flushing, due to serotonin production while atypical carcinoid syndrome is characterized by prolonged

flushing, bronchospasm, and hypotension due to histamine[13]. This syndrome can cause carcinoid heart disease, involving the right-sided heart valves and possibly leading to right heart failure, that increase the morbidity and mortality of the patients[14].

Insulinomas are rare pancreatic neoplasms causing hypoglycemia, especially while fasting or during exercise, with inappropriate insulin suppression. The Whipple's triad, the contemporary presence of symptoms of hypoglycemia, low blood glucose levels, and relief of symptoms by intake of glucose, is suggestive of insulinoma[15].

Glucagonomas are rare pancreatic neoplasms secreting glucagon causing diabetes mellitus, associated with the 4D syndrome (dermatosis/necrolytic migratory erythema, depression, deep vein thrombosis, diarrhea)[16].

Somatostatinomas are tumors located in the pancreas or duodenum able to produce somatostatin. The clinical presentation is diabetes mellitus, diarrhea, steatorrhea, gallbladder disease, hypochlorhydria, and weight loss[17].

In Zollinger-Ellison Syndrome, there is an overproduction of gastrin causing gastric acid hypersecretion. Patients could develop recurrent peptic ulcers, gastroesophageal reflux disease, and chronic diarrhea[18].

The Verner-Morrison syndrome, VIPoma, is characterized by the contemporary presence of watery diarrhea, hypokalemia, and achlorhydria (WDHA syndrome) and is caused by an excess of vasoactive intestinal peptide[19].

NENs can also produce peptides able to cause endocrine ectopic syndromes, such as acromegaly (growth hormone production), syndrome of inappropriate antidiuresis, SIAD (ADH production), Cushing syndrome (ACTH production), hypercalcemia (PTH related peptide production)[20].

In non-functioning tumors, symptoms are usually caused by mass effect or metastatic spread, and therefore these tumors can remain asymptomatic for a long time, delaying the diagnosis. Accordingly, epidemiological studies demonstrate that a significant proportion

of patients presented with metastatic disease (28% at diagnosis)[3] with a delay in the diagnosis that can reach 4 years[21]. Symptoms are often not specific, such as pain and nausea, and can mimic many other disorders[12].

Histological Classification

Initially, neuroendocrine tumors were called "carcinoid", a word that derives from the German "Karzinoid", first reported in 1907, used for identifying a neoplasm with a better prognosis than adenocarcinomas[22]. Since that moment, important changes have been done in the classification of NENs, and WHO created the first classification in 2000.

The last WHO classification, for GEP NENs, published in 2022, recommended classifying NENs according to morphology: well-differentiated, known as NETs, and poorly differentiated, known as NECs. Grading (grade 1,2,3) is assessed using the mitotic index and Ki67 labeling index, as summarized in Table 1. Mixed neuroendocrine-non-neuroendocrine neoplasms, MiNEN, have, for definition, at least 30% of cells for the neuroendocrine and non-neuroendocrine components. The neuroendocrine components can rarely be well differentiated (previously known as MANET), and more frequently poorly differentiated (previously known as MANEC)[23,24].

	Morphology	Grade	Mitotic index	Ki67
Neuroendocrine tumors	Well-differentiated	1	<2/10HPF*	<3%
	Well-differentiated	2	2-20/10HPF	3-20%
	Well-differentiated	3	>20/10HPF	>20%
Neuroendocrine carcinomas, small cell type	Poorly differentiated	3	>20/10HPF	>20%
Neuroendocrine carcinomas, large cell type	Poorly differentiated	3	>20/10HPF	>20%
Mixed neoplasia (MiNEN)	Well or poorly differentiated			

Table 1. Histological classification of GEP-NENs, according to WHO 2022 classification. *High Power Fields (magnification, 40x). MiNEN: Mixed neuroendocrine-non-neuroendocrine neoplasms.

Lung Neuroendocrine Neoplasms are classified according to the WHO classification of 2022 in 4 different categories, as summarized in Table 2: the well-differentiated form, typical carcinoid, and atypical carcinoid, and the poorly differentiated ones, large-cells neuroendocrine carcinoma and small cell neuroendocrine carcinoma (previously called

microcitoma)[25]. Differently from GEP-NENs, the grade is based on mitotic index (without considering Ki67 LI) and necrosis. However, a consensus of the European Neuroendocrine Tumors Association reported that the ki67 labeling index could have a prognostic role and could help in differentiating typical from atypical carcinoids and well-differentiated forms from neuroendocrine carcinomas[26]. Last WHO classification included a new category, called carcinoids/NETs with elevated mitotic counts and/or Ki67 proliferation index, thus inserting Ki67 labeling index in the classification[25].

	Morphology	Mitotic index	Necrosis
Typical carcinoid/NET grade 1	Well-differentiated	<2/10HPF*	Absent
Atypical carcinoid/NET grade 2	Well-differentiated	2-20/10HPF	Present (often punctate)
Carcinoids/nets with elevated mitotic counts and/or ki67 proliferation index	Well-differentiated	> 10/10HPF Ki67>30%	
Large cell neuroendocrine carcinoma	Poorly differentiated	>10/10HPF	Virtually always present (often large zone)
Small cell neuroendocrine carcinoma	Poorly differentiated	>10/10HPF	Often present (often large zone)

Table 2. Classification of lung neuroendocrine neoplasia, according to WHO 2022. *High Power Fields (magnification, 40x).

Immunohistochemistry can be used on metastatic tissue samples to suggest the primary site of a neoplasm. Only in well-differentiated tumors, some transcription factors are differentially expressed: CDX2 in the bowel, TTF1 in the lung, and PAX8, ISL1, and PDX1 in the pancreas[27].

Staging systems

The eighth edition of the TNM classification, published by the Union for International Cancer Control and the American Joint Committee on Cancer (UICC/AJCC) includes a classification of the well-differentiated GEP-NETs (Grade 1 and 2). Poorly differentiated carcinomas are, on the contrary, not included and must be classified according to the classification of the organ in which they arise. Only for GEP-NENs also ENETS published a staging system for foregut (gastric, duodenum, and proximal jejunum), midgut (jejunum, ileum, appendix), and hindgut NENs (colon and rectum). Both classifications are used in

clinical practice[28]. On the contrary, only the TNM classification (eighth edition) for carcinoma of the lung is used for lung NENs[29].

Pathogenesis and molecular mechanisms

Neuroendocrine neoplasms can be sporadic or occur in the context of genetic syndromes. The most frequent is multiple endocrine neoplasia type 1, characterized by inactivating mutations in the *MEN1* gene, which encoded for MENIN. Clinically, MEN1 patients developed pancreatic NETs in addition to pituitary tumors and primary hyperparathyroidism. MEN2, caused by a mutation in *RET* gene, is associated with thyroid and adrenal NETs and hyperparathyroidism. Men 4 is caused by the inactivation of *CDKN1B* gene, encoding for p27. It is characterized by pituitary tumors and hyperparathyroidism, associated with pancreatic, gastric, and lung NENs[30]. Other genetic syndromes associated with a higher risk of developing NENs are Von Hippel Lindau, Neurofibromatosis type 1, and Tuberous Sclerosis[31].

Genetic NENs also offer the possibility to study the genetic alterations causing sporadic NENs. In fact, since 2010 low or absent expression of MENIN has been found in over 70% of lung and pancreatic sporadic NETs[32]. Translating the knowledge derived from genetic NET, it was possible to identify two main pathways involved in sporadic NET development: cyclin-dependent cell cycle regulation (MEN1, MEN4) and the PI3K/mTOR pathway (MEN1, VHL, NF1, TS)[33].

The use of next-generation sequencing (NGS) has allowed the identification of genetic alterations across the whole genome or exome. The first study in pancreatic NETs confirmed the crucial role of the mTOR pathway. The Authors identified a high prevalence of MEN1 mutation and the presence of inactivating mutation in *TSC2* and *PTEN* genes, encoding for proteins implicated in mTOR pathways[34], as well as two novel genes, *DAXX*/*ATRX*, which mutations are associated with alternative lengthening of telomeres (ALT)[35]. Subsequent studies demonstrated genetic alteration also in DNA damage repair, telomere alteration, cell cycle, and chromatin remodeling/histone methylation[33]. In addition, also epigenetic

alterations have been described in pancreatic NETs, such as methylation of *RASSF1*, *HIC1* (hypermethylated in cancer 1), *CDKN2A*, *MGMT*, and *VHL*[36].

Overall, four are the altered pathways in pancreatic NETs: DNA damage repair, chromatin remodeling, telomere length alteration, and the PI3K/mTOR signaling pathway[36].

In small intestinal NETs, the overall genetic landscape is less clear than in pancreatic NETs. The first study revealed somatic copy number alterations that determined the deregulation of two pathways: TGF-β/Wnt and PI3K/mTOR[37]. Some following studies demonstrated the presence of mutations or deletions of the *CDKN1B* gene, involved in cell cycle regulation[38], mutations in APC gene[39], and chromosome 18 loss[40]. In small intestinal NETs also, the role of epigenetic mutation has been extensively studied. Hypermethylation of the promoter of many genes, including *RASSF1A* has been described[33]. Overall, it is more difficult to summarize the altered pathways as in pancreatic NETs, but they included the mTOR pathway, cyclin-dependent cell cycle regulation, the Wnt pathway, and DNA single-base repair[33].

1.2 Diagnostic and prognostic circulating markers in NENs

In clinical practice, the most reliable prognostic markers are tumor differentiation and grade[41]. In addition, also other histological markers can predict the prognosis of the patients, such as DAXX/ATRX, Cytokeratin 19, INSM1, and c-kit[42]. Morphological imaging, as positron emission tomography/computed tomography (PET/CT) with 18F-FDG has demonstrated a clear prognostic role, been correlated to overall survival[43]. However, also tumors with the same histological and morphological characteristics can behave differently, and there is a medical need to rely on easy-to-execute and repeatable analysis, mostly circulating biomarkers, which can predict prognosis and tumor response.

Monoanalyte diagnostic and prognostic biomarkers

Some circulating markers are used for the diagnosis and follow-up of NENs. Functioning NENs cause clinical syndromes through the production of a specific hormone, which can

usually be used for the diagnosis and for assessing treatment response[44]. Table 3 summarized the biomarkers used in clinical practice.

Syndrome	Circulating biomarkers and remarks
Carcinoid syndrome/carcinoid heart disease	5-HIAA / NTpro-BPN *Especially if 5-HIAA is doubled the upper limit of normal*
Insulinoma	Insulin *hypoglycemia (glucose<55 mg/dl) with insulin ≥3,0 μU/ml*
Gastrinoma (zes)	Gastrin *In case of acid gastric pH (<2)*
Vipoma	VIP
Glucagonoma	Glucagon
Somatostatinoma	Somatostatin
NETs causing ectopic syndromes	PTHrp, ACTH, CRH, GHRH
Pheocromocytoma/paraganglioma	Catecholamines and metanephrines
Medullary thyroid carcinoma	CT

Table 3. Main markers of functioning neuroendocrine neoplasms. Adapted from Oberg et al[45] and Kormarnicki et al[46]. ZES: Zollinger Ellison Syndrome; 5-HIAA: 5-hydroxyindoleacetic acid; NTpro-BPN: N-terminal prohormone of brain natriuretic peptide, VIP: vasoactive intestinal peptide; NETs: neuroendocrine tumors, PTHrp: parathyroid hormone related peptide; ACTH: adrenocorticotropic hormone, CRH: corticotropin releasing hormone, GHRH: growth hormone releasing hormone; CT: calcitonin.

However, only a small proportion of NENs are functioning, and in non-functioning NEN general tumor markers, such as chromogranin A (CgA) and neuron specific enolase (NSE), are commonly used but their sensibility and specificity are quite low.

Chromogranin A is s an acidic glycoprotein of the secretory granules of most physiologic and neoplastic neuroendocrine cells[47]. It is the most used non-specific neuroendocrine tumor marker. It also has a prognostic role, since its levels are inversely correlated to overall survival[48] and tumor burden[49] and can predict treatment response[50,51].

A recent meta-analysis on the utility of chromogranin A assessment in bronchopulmonary NENs demonstrated a diagnostic sensitivity of 34.5 ± 2.7% with a specificity of 93.8 ± 4.7% in typical and atypical carcinoids and 59.9 ± 6.8% and 79.4 ± 3.1% in small cells lung cancers. Evidence on the role of this marker in the evaluation of overall survival derives only from 6 retrospective studies (2 for carcinoids, 4 for small cell lung cancers) and no studies evaluated the role of this marker in the prediction of treatment response and in detecting residual

disease after surgery[52]. In the diagnosis of GEP-NENs, a meta-analysis of 13 studies evidenced a pooled sensitivity of chromogranin A of 73% (95% confidence interval: 71 - 76) and a specificity of 95% (95% confidence interval: 93 - 96)[53]. A following meta-analysis demonstrated that chromogranin A is more reliable when used to monitor disease progression and response to treatment, in comparison with diagnosis[54]. Despite the meta-analysis results, it is known that many conditions can alter chromogranin A levels, reducing its specificity, such as chronic atrophic gastritis, treatment with proton pump inhibitors or glucocorticoid, and impaired renal function[55]. Finally, also there is no recognized international standard for chromogranin A assay as well as no reference intervals[56]. In conclusion, even if this is the most useful circulating general tumor marker in patients with NETs, it has substantial limitations[57].

Neuron-specific enolase is an important biomarker only for high grade tumors with a sensitivity of 63% and 62% in large and small cell neuroendocrine carcinomas[58]. A recent meta-analysis demonstrated that neuron-specific enolase levels are associated with worse progression free survival and overall survival in small cell neuroendocrine carcinomas[59]. The prognostic role of neuron-specific enolase has been confirmed also for GEP-NENs[51].

Other monoanalyte biomarkers have been studied but are not part of standard clinical practice. Pacreastatin is produced by enzymatic cleavage of chromogranin A, which has the same sensibility and specificity as its precursor, but it is not affected by proton pump inhibitor assumption[60]. Pancreatic polypeptide has low sensitivity for GEP-NETs (18-63%) if used alone[61], but shows a higher sensitivity when used in association with chromogranin A (84-96%)[62]. Neurokinin A seems a good candidate marker for small intestinal NETs, and its levels are associated with poor prognosis[63]. Interestingly, the serum neutrophil-lymphocyte ratio has demonstrated a correlation with stage and recurrence after surgical resection in large and small cell neuroendocrine carcinomas[64,65]. In gastric neuroendocrine neoplasms, the neutrophil-lymphocyte ratio correlated with relapse free survival and overall survival[66]. On the contrary, using data from the randomized double-blind

CLARINET study the neutrophil-lymphocyte ratio was not a predictor of progression free survival in pancreatic and intestinal NETs[67].

New diagnostic and prognostic circulating biomarkers

Starting from the great heterogeneity which characterizes NENs, many experts agreed that multianalyte panels can be better biomarkers in NENs field[57]. In recent years, liquid biopsy has received growing attention in oncology. It is a technique used to identify and characterize a neoplasm through the analysis of patients' blood samples[68]. Many are the advantages of liquid biopsy. First, samples are easy to obtain, with a lower complication rate than classical histological biopsy[69]. Second, the procedure is repeatable, allowing to assess if the molecular features of the neoplasm have changed[70]. Third, it can overcome tumor heterogeneity, better reflecting different tumor localizations[71,72]. Finally, liquid biopsy could also play a role in patients at remission, since the positivity of the liquid biopsy could detect the relapse earlier than classical radiological imaging, unmasking the minimal residual disease[73]. On the other hand, many are the disadvantages of liquid biopsy: procedures are not standardized[74]; not all tumors can release components in the bloodstream[75]; and only a part of the circulating materials came from neoplastic cells, reducing the accuracy of the analysis[68,69]. The term liquid biopsy encloses the research of nucleic acids (RNA and DNA), circulating tumor cells (CTC), exosomes, and tumor-educated platelets (TEPs)[69], but studies in NENs are scarce.

The most studied type of liquid biopsy applied to NENs is the NETest, a standardized and reproducible analysis for the diagnosis of NENs starting from the isolation of messenger RNA circulating in the blood. After the production of cDNA, NETest evaluates the expression of 51 genes through a real-time polymerase chain reaction. An algorithm-based analysis generates a clinical score varying from 0 to 100%[76]. Initially evaluated in GEP-NENs, further studies confirmed the diagnostic accuracy also in lung NENs[77] and pheochromocytomas/paragangliomas[78]. A meta-analysis including ten studies concluded that the diagnostic accuracy is very high (95%-96%)[79]. Our group has performed a systematic review on the prognostic and predictive role of NETest, highlighting the possibility of this

analytical examination to differentiate stable from progressive disease (with a cut-off of 40%) at the moment of the blood sample and to identify minimal residual disease after surgery (with a cut-off of 20%) and therefore to predict recurrence. The role of NETest in other settings, such as the prediction of tumor progression or response to treatment, needs to be confirmed by other studies[80]. The main limitation for the spread of NETest in clinical practice is the lack of data on cost-effectiveness and the low number of Laboratories able to perform the analysis[46].

Circulating tumor DNA (ctDNA) are portions of tumor-derived DNA that are released in the bloodstream after apoptosis[81]. High levels of ctDNA have been described in pancreatic and small intestinal NENs[82]. The main limitation of this approach in NENs is the risk of false negative results due to the variable amount of ctDNA that can be released by the tumors; for this reason, it is more reliable in the case of high-grade neoplasms[83].

MicroRNAs (MiRNAs) are non-coding RNAs able to promote or suppress gene expression at a post-transcriptional level. MiRNAs have been studied in many neoplasms, but few data are available in NENs[46]. A systematic review demonstrated that the expression of MiRNAs varies according to primary tumor sites, and only miR-21 and miR-133a have been detected in both small intestinal and pancreatic NENs[84]. The study by Melone *et al.* was the first to identify a set of circulating MiRNAs able to be used for NEN diagnostics[85]. As in other multianalyte markers, the clinical validation of MiRNAs needs the development of a mathematical algorithm for improving its accuracy[86].

CTC are neoplastic cells that enter the bloodstream. This phenomenon is at the base of the metastatic process but has also been used for the diagnosis and characterization of many types of cancer[87]. CTC were identified in a small percentage of pancreatic and small intestinal NENs (all metastatic) in 2011[88]. Further studies also demonstrated the association of CTC with progression free survival and overall survival[89,90] and with treatment response[91]. Available data are heterogeneous and scanty and, also because NENs can be indolent, the applicability of CTC in NENs needs to be confirmed[46].

It has been demonstrated that the interplay between platelets and tumor cells is involved in tumor growth and dissemination[92]. Moreover, platelets can take up tumor-derived secreted membrane vesicles containing RNAs, becoming TEPs[93]. It is therefore possible to isolate platelets to have access to tumor RNA, a potential biomarker for cancer diagnostics. circular RNAs (circRNAs) is a non-coding RNA, originating from back splicing, characterized by a covalently closed loop structure due to the bind of 5′ splice site with upstream 3′ splicing site of a pre-mRNA molecule, gaining stability. circRNAs play many roles: inhibitors of microRNA or protein (acting as 'sponges'), regulators of protein function through the binding of specific proteins to multiple circRNAs (acting as a molecular reservoir of proteins), and more rarely, being translated (coding circRNA)[94]. In non-neuroendocrine tumors, the evaluation of circRNA from TEPs can discriminate patients affected by neoplasms from healthy subjects and gives the possibility to identify the primary tumor histotype and detect possible predictors of treatment response[95]. This innovative approach to cancer detection has not yet been transferred to the NEN field.

1.3 Angiogenesis in NENs

NENs are highly vascularized neoplasms. Intratumoral vascular density is higher in well-differentiated tumors than in poorly differentiated forms, probably because NETs resemble the normal endocrine glands, which need vessels for hormone secretion[96]. This characteristic, known as the neuroendocrine paradox, differentiates neuroendocrine tumors from other neoplasms, in which the increase in vascularization is usually associated with a more aggressive disease[97]. Tumor angiogenesis is a complex mechanism by which the tumors promote the formation of new vessels from the sprouting of pre-existing vessels, to facilitate tumor growth. In addition, blood vessels can present aberrant characteristics, such as fenestrations, discontinuous basement membrane, and lack of pericyte coverage, that favors cell extravasation and tumor metastatization[98]. In physiological conditions, pro-angiogenic and anti-angiogenic factors are closely balanced; on the contrary, in the presence of neoplasm, there is an imbalance in pro-angiogenic factors produced by the neoplastic cells and, in turn, endothelial cells produce anti-apoptotic factors, an event called

angiogenetic switch[99]. The main molecular pathways involved in NENs angiogenesis are vascular endothelial growth factor (VEGF) and its receptors (VEGFR); fibroblast growth factor (FGF) and its receptor (FGFR) and platelet derived growth factor (PDGF) and its receptor (PDGFR)[100].

It has been demonstrated that NETs are able to produce VEGF, at a higher concentration than NECs, and VEGFR is expressed by the tumor and by the endothelial cells[101]. Animal evidence demonstrates that VEGF plays a role in the tumorigenesis of pancreatic tumors since selective knock-out for VEGF can prevent the growth of pancreatic NETs[102].

FGF/FGFR pathway exerts direct effects on angiogenesis, promoting vessel formation by endothelial cell migration, proliferation, and differentiation. Studies have confirmed the expression of FGF and its receptors in NENs[103] and the FGF system seems also implicated in gastrointestinal fibrosis associated with NENs[104]. FGF pathway acts also indirectly since it is able also to enhance other mediators, such as VEGF and angiopoietins[105]. A pre-clinical study in a mouse model of pancreatic NETs demonstrated that the inhibition of VEGFR2 can inhibit the angiogenic switch but the resistance to this molecule, which appears in late-stage tumors, is due to the activation of FGF pathways[106]. In this context, the contemporary blocking of FGF and VEGF pathways can overcome the development of tumor resistance[107].

The PDGF/PDGFR can enhance angiogenesis through pericytes recruitment and the PDFG knock out significantly delayed pancreatic NETs development[98]. The expression of PDGFR has been demonstrated in NENs: PDGFR-α is expressed by tumor cells and is correlated with the grade, while PDGFR-β is expressed by stromal cells and pericytes and is correlated to microvascular density[108].

More recent evidence demonstrated the contribution of Angiopoietins (ANG) and their receptors in NENs. In physiological conditions, the ANG1 binds Tie2, a tyrosine kinase receptor expressed on the endothelial cells and macrophages, promoting blood vessel stability[109]. ANG2 acts as an antagonist of the receptor TIE2, promoting angiogenesis and vascular instability[110] and facilitating the response of the endothelium to other cytokines[109].

High levels of ANG2, acting through β1 integrin activation are also one of the mechanisms responsible for the loss of cell-matrix contact, pericyte detachment, and vascular leakage[111]. Some studies have confirmed higher levels of circulating angiopoietins in the serum of patients with NENs compared with healthy controls, also demonstrating the expression of ANG2 and TIE2 mRNA by neoplastic cells, suggesting a paracrine action[112]. The overexpression of ANG2 has been demonstrated as a mechanism of resistance of VEGF inhibitors[113].

A growth factor selective for the endocrine glands, called endocrine-gland-derived vascular endothelial growth factor or prokineticin1 (PROK1), has been identified in 2001. It induces proliferation, migration, and fenestration in capillary endothelial cells derived from endocrine glands[114]. The PROK1 and 2 and their receptors (PROKR) 1 and 2 are involved in the angiogenesis of many kinds of cancers, including some in the endocrine organs, such as ovarian carcinoma, prostate, and testicular cancer[115]. In the presence of hypoxia, the levels of PROK1 and 2 increase, stimulating angiogenesis[116]. Moreover, the upregulation of PROK2 plays a role also in acquired drug resistance to VEGF inhibitors[117].

The crucial role of angiogenesis is testified also by the role of angiogenetic treatments in NENs. Sunitinib, a muti-target antiangiogenetic treatment, is already approved for the treatment of pancreatic NETs[118]. Trials have evaluated the use of antiangiogenic drugs in NENs, such as the recent SANET-P and SANET-EP which demonstrated the efficacy of surufatinib, an anti-angiogenic drug, in pancreatic[119] and extra-pancreatic NENs[120]. Bevacizumab, a monoclonal antibody blocking VEGF, in association with chemotherapy (FOLFOX and FOLFIRI) was effective both as a first- or second-line strategy in poorly differentiated NEC[121].

2. AIM OF THE STUDY

The aim of this study was to evaluate the levels of circulating angiogenic markers in patients affected by pulmonary and GEP NENs, comparing these patients with healthy controls and confirming the expression of these markers by immunohistochemistry. Moreover, we assessed the prognostic values of these markers through their association with NENs characteristics, such as morphology, staging, and disease status (both at baseline and during follow-up). In a subgroup of well-differentiated GEP-NETs (grade G1 and G2), naïve to treatment, we also evaluated, for the first time, the expression profile of circRNA from TEPs, exploring the potential roles of this kind of liquid biopsy.

3. MATERIALS AND METHODS

3.1 Study design and population

This was a prospective observational case-control study, performed at one Institution. Patients were selected from the outpatient endocrinology clinic of the Department of Experimental Medicine at "Sapienza" University of Rome, in the Neuroendocrine Tumor task force Unit (NETTARE) of the Umberto I University Hospital.

The inclusion criteria for the study group were:

- Diagnosis of pulmonary or GEP NEN confirmed by histological examination (through biopsy or surgery);
- Age between 18 and 80 years;
- Signed informed consent.

The inclusion criteria for the control group were:

- Diagnosis of benign thyroid disease;
- Age between 18 and 80 years;
- Signed informed consent.

All consecutive patients who met the inclusion criteria were enrolled in the study.

Exclusion criteria for both groups included: severe chronic kidney disease (stage 4–5); clinical or laboratory signs of significant respiratory, cardiological, and hepatobiliary disease; other non-neuroendocrine malignancies.

A limited subgroup of patients was also included in a proof-of-concept pilot study. To be included, patients should have a new diagnosis of well-differentiated GEP NET, grade G1 or G2, naïve to any medical treatment. The study flowchart is reported in Figure 1.

All patients provided written informed consent to study participation. The study was approved by the local ethic committee board at Sapienza University of Rome (reference number 5917) and conducted in accordance with the Declaration of Helsinki.

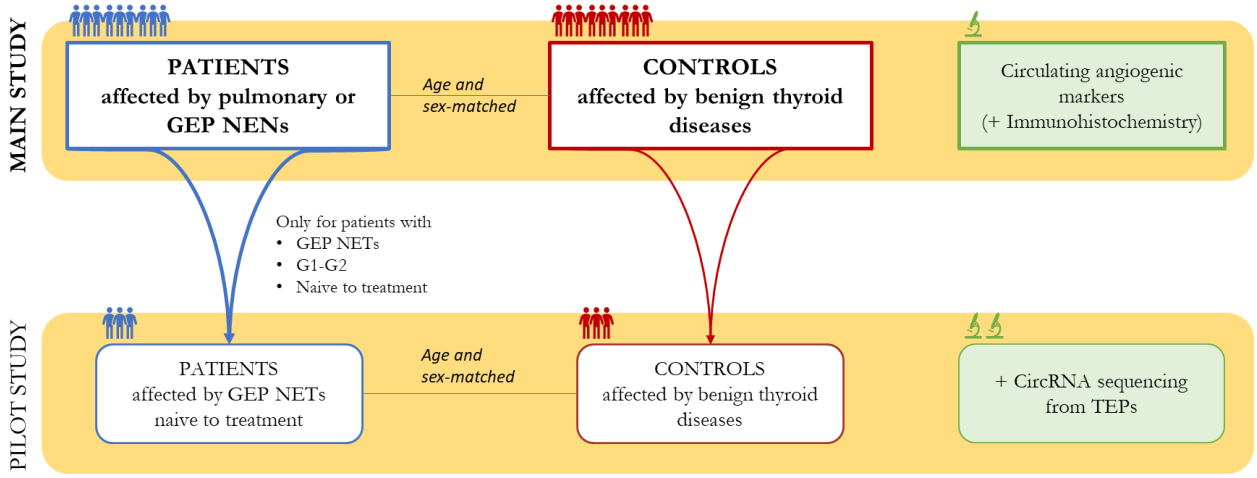

Figure 1. Study flowchart. GEP: gastro-entero-pancreatic, NET: neuroendocrine tumor, CircRNA: circular RNA; TEPs: tumor-educated platelets.

3.2 Study procedures

Blood samples were taken from a peripheral vein in a fasting state at 8:00 in the morning for all the patients and controls included in the main study at one time point. Patients were subsequently treated and evaluated according to current clinical practice and these data were collected to obtain information on disease status after enrollment. Only for patients included also in the pilot study, blood samples were taken also after three months of standard treatment (surgery or medical treatment with somatostatin analogs, SSA).

Serum angiogenesis markers

The evaluation of serum angiogenesis mediators was performed in serum by ELISA, using commercially available kits: human soluble TIE2 ELISA Kit (Abcam, Cambridge UK, human Prokineticin 1 (PROK1/EGVEGF) ELISA Kit (Cusabio, Houston, TX, USA); human Prokineticin 2 ELISA Kit (Cusabio, Houston, TX, USA, detection range: 6.25–400 pg/ml); human ANG1 ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA); human ANG2

ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA). According to the manufacturer's instructions, all experimental analyses were performed by duplicate.

Immunohistochemistry

GEP-NENs tissue specimens were fixed in 10% neutral buffered Formalin (Sigma-Aldrich, Saint Louis, MO, USA) and embedded in Paraffin (Bio Optica, Milano, Italy). For the analysis, the five-micrometer sections were obtained with the HM355S Microtome (Thermo Fisher Scientific, Waltham, MA, USA) and were subsequently de-waxed, re-hydrated, and processed for immunohistochemistry using Multivision Polymer detection System (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Antigen retrieval was obtained by microwaving sections in 10mM Sodium Citrate pH 6.0+0.05% Tween for 10 min. The sections were incubated overnight with primary antibodies (ANG2, Abcam #ab153934; Chromogranin A, Thermo Scientific #MA5-13096) at +4 °C. Before mounting, slides were counterstained with Hematoxylin (Sigma-Aldrich, Saint Louis, MO, USA). Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Inc., Thornwood, NY, USA) was used for imaging acquirement.

Platelets isolation and RNA extraction

EDTA-coated purple-capped Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) have been used for whole blood collection. Tubes were centrifuged at 120× g for 20 minutes at room temperature (RT) for separating platelet-rich plasma from nucleated blood cells. Plasma was then centrifuged at 360× g for 20 min at RT to pellet platelets, which were resuspended in RNAlater (Thermo Scientific, Waltham, MA, USA) and stored at −80 °C until analysis. Total RNA isolation was performed using miRNeasy Micro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. On column DNase digestion was performed during extraction. RNA quality was assessed using RNA 6000 Picochip - Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Only samples with a RIN-value greater than 7 and/or distinctive rRNA curves were included for analysis.

Library Construction

SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio Inc., Shiga, Japan) was used for library preparation, according to the manufacturer's recommendations. Library quality and quantity were assessed with Qubit 2.0 DNA HS Assay and Tapestation D1000 Assay (Agilent, Santa Clara, CA, USA). Final libraries were quantified using the QuantStudio ® 5 System (Applied Biosystems, California, USA) before equimolar pooling based on qPCR QC values.

RNA Sequencing and data analysis

An Illumina® NovaSeq (Illumina, San Diego, CA, USA) was used to perform sequencing, with a read length configuration of 150 paired-ends for 120M paired-ends reads (60M in each direction) per sample. Run files were demultiplexed using bcl2fastq Software v2.20 (Illumina, San Diego, CA, USA). A quality check was carried out on the raw data, to remove low-quality portions of NGS reads. The trimming step was performed by setting the minimum length to 35 bp and the quality score to 25 using the BBDuk Software. The quality before and after trimming was evaluated with the software FASTQC.

HISAT-v2.1.0 was used to map the sequenced reads against an in-house generated from the University of California Santa Cruz (UCSC) reference, which is based on the human reference genome (hg38). After sorting for name and chromosome, followed by indexing with Samtools-v1.9, the consistency and the quality of .bam files were checked using Integrative Genomics Viewer-v2.5.3.

CircRNAs annotation and identification of differentially expressed circRNAs

CircRNAs annotation was carried out with CIRCexplorer2 Software. The high-quality readswere mapped on the human reference genome (hg38) using STAR. The chimeric reads were then processed with CIRCexplorer2 providing the official hg38 annotation (Ensembl release 105). The identification of the differentially expressed circRNAs was performed with the package edgeR, the threshold for significance is FDR≤0.05. Only the circRNAs with at least 3 reads in each replicate separated per group were considered. A second step of

annotation was performed via blast search against the circBase database[122]. The blast search was conducted against the Homo sapiens genome version hg19.

Analysis of the differentially expressed circRNAs between groups (NET patients and controls, and between NET patients before and after treatment) is currently ongoing, and are performed in R using the edgeR package[123].

The workflow of circRNA sequencing from TEPs is summarized in Figure 2.

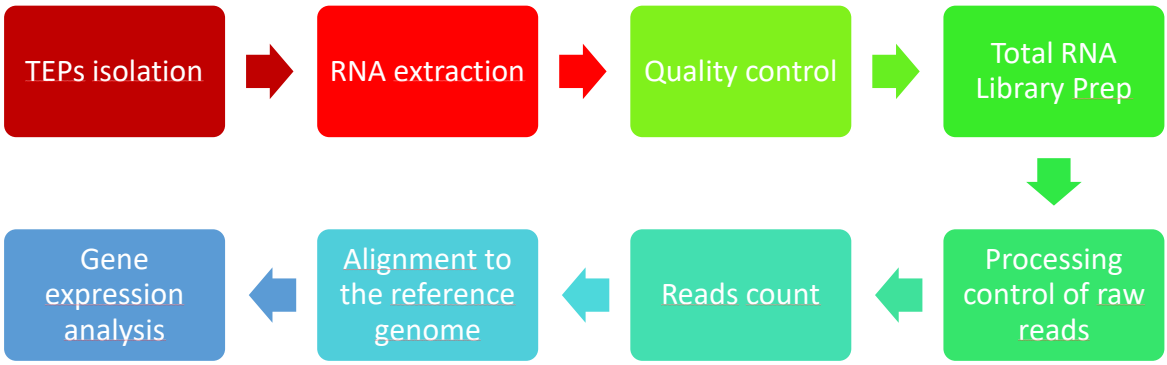

Figure 2. Workflow of circRNA sequencing from TEPs. TEPs: tumor-educated platelets.

3.3 Data collection

The following data were collected for all patients and controls enrolled in the study: age at diagnosis, sex, body mass index (BMI), smoking habits, and comorbidities. For the study group, we collected also: the tumor primary site, staging, morphology and grading, treatment strategy, disease status (partial response, stable disease, progressive disease) at the moment of the blood sample and during the follow-up, and treatment strategies.

3.4 Statistical analysis

Sample size calculation

For the main study, the primary efficacy variable was the difference in ANG2 levels between patients and controls; therefore, the null hypothesis was that there was no difference in ANG2 serum levels between groups. For the rejection of the null hypothesis, a significance level of 0.05 was required. The tests were two-tailed, so the effects were interpreted in both directions. In the healthy population, ANG2 was 1.7 ± 0.9 ng/ml, while in NEN patients ANG2 was 3.6 ± 2.4 ng/ml (expressed in average value ± standard deviation, SD)[124]. A sample size of 29 patients for each group achieved 85% power to detect a difference in ANG2 levels between groups with a significance level (alpha) of 0.05 using a two-sided one-sample t-test. No sample size calculation was performed for the proof-of-concept pilot study.

Statistical analysis

Categorical variables are expressed as frequencies and percentages. Continuous variables are reported as mean ± standard deviation for normally distributed variables and as median (25th–75th percentiles) for non-normally distributed variables. The normality of distribution was assessed by Shapiro–Wilk test and homoscedasticity by Levene's test or Kolmogorov–Smirnov test as appropriate. The difference between the binomial proportions of a dichotomous-dependent variable was assessed by $\chi 2$ test for homogeneity. The difference in continuous variables between groups was evaluated by Student's t-test (for normally distributed variables), Mann–Whitney test (for non-normally distributed variables, in case of two groups), or Kruskal–Wallis test (for non-normally distributed variables, in case of several groups). A p value of less than 0.05 was considered significant. All statistical analyses were performed using SPSS for Windows, version 20.0 (SPSS, Inc.).

4.1 RESULTS

In the main study, we enrolled 46 consecutive patients affected by histologically confirmed pulmonary or GEP NEN. The mean age was 66.1 ± 12.5 years old; males were 54.4%. Regarding the primary site of NENs, 27 (54.4%) of patients had GEP neoplasms (14 pancreatic, 9 small intestinal, 2 large intestinal) and 21 (45.7%) had pulmonary neoplasms. According to the WHO classification of NENs[24], most GEP-NENs were histologically well differentiated (84.0%), while most lung NENs were poorly differentiated carcinomas, both large and small cell types (76.2%). Overall, tumor grading was G1 (39.1%), G2 (17.4%), NET G3 (2.2%), and NEC (41.31%). Locally advanced or metastatic disease (TNM stage III or IV) represented 60.9% of cases. At the enrollment in the study, only some patients (17.4%) were naive to treatment, while others had received at least one cycle of standard therapy (first-line therapy) consisting of SSA for well-differentiated NETs (43.5%) and chemotherapy with cisplatin and etoposide for poorly differentiated NECs (39.1%). Thirteen patients also underwent surgical intervention. The main age of the control group was 61.6± 7.9, with 65.5% of male subjects, without statistically significant difference between patients and controls. In addition, no difference was found between groups regarding the frequencies of possible confounding factors, such as BMI, smoking habits, arterial hypertension, and diabetes mellitus. The main baseline characteristics of NENS and controls are summarized in Table 4 and Table 5.

	NENS (46)	**CONTROLS (29)**	**p VALUE**
Age (years)	66.0 ± 12.5	61.6 ± 7.9	0.06[a]
Sex (m/f, n)	25/21	19/10	0.34[b]
BMI (kg/m²)	24.9 ± 5.0	25.8 ± 3.2	0.57[a]
Smoking habits	11 (26%)	7 (24%)	0.85
Arterial hypertension	6 (13%)	3 (10%)	0.72
Diabetes mellitus	2 (4%)	1 (3%)	0.85

Table 4. Baseline characteristics of both groups. NENs: neuroendocrine neoplasms, M: male, F: female, BMI: body mass index. Data are expressed as mean ± standard deviation or relative frequency (percentage). a: Student's t-test, b: $\chi 2$ test.

NEN characteristics	
Site	
- GEP	25/46 (54.4%)
- Pulmonary	21/46 (45.7%)
Grade	
- G1	18/46 (39.1%)
- G2	8/46 (17.4%)
- NET G3	1/46 (2.2%)
- NEC	19/46 (41.3%)
Stage	
- Localized (TNM stage I, II)	18/46 (39.1%)
- Locally advanced or metastatic (TNM stage III, IV)	28/46 (60.9%)
Therapy	
- Naïve	8/46 (17.4%)
- SSA	20/46 (43.5%)
- CHT	18/46 (39.1%)

Table 5. Patients' baseline characteristics. NEN neuroendocrine neoplasms, M male, F female, BMI body mass index, GEP gastro-entero-pancreatic, G grade, NET neuroendocrine tumor, NEC neuroendocrine carcinoma, SSA somatostatin analogs, CHT chemotherapy. Data are expressed as relative frequency (percentage).

4.1 Comparison of angiogenic factors between patients and controls

We first compared the circulating levels of angiogenic factors in patients and controls. The levels of both angiopoietins were significantly higher in patients than in controls. ANG2 levels were 4.0 (2.3–6.4) ng/ml in patients and 1.6 (0.6–2.8) ng/ml in controls ($p < 0.001$); ANG1 levels were 61.5 (34.3–94.9) ng/ml in patients and 29.3 (19.4–45.6) ng/ml in controls ($p < 0.001$). No statistically significant difference was found in ANG1/ANG2 ratio. The soluble ANG receptor, sTIE2, was also higher in patients than controls, respectively 52.8 (37.3–119.4) and 24.5 (21.4–34.4) ng/ml, $p < 0.001$.

Regarding PROKs, no difference was found in PROK1 levels between groups while PROK2 levels were higher in patients than controls: 28.6 (28.2–29.7) *vs* 28.1 (28.0–28.5) pg/ml, $p < 0.001$. Table 6 and Figure 3 summarized the differences in the evaluated angiogenic markers between groups.

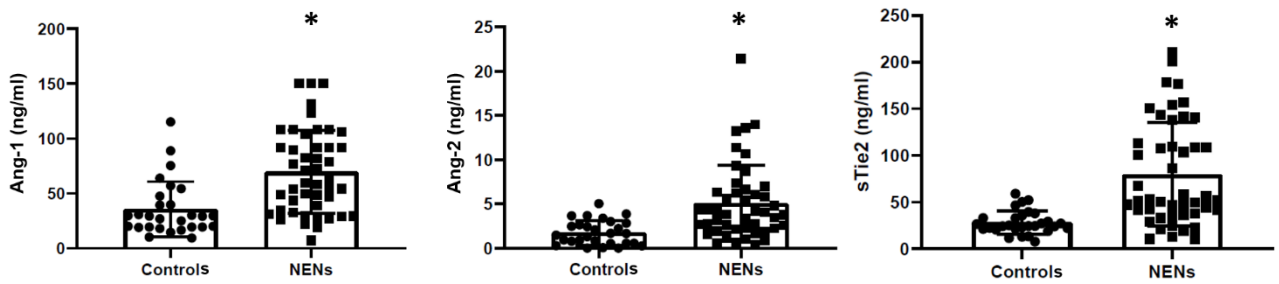

Figure 3. Circulating angiogenic factors levels in patients affected by NENs and controls. Values are reported as mean ± standard error of mean. NEN neuroendocrine neoplasms, ANG: angiopoietin, sTIE2: soluble TIE2.

	NENS (46)	CONTROLS (29)	p VALUE
ANG2 (ng/ml)	4.0 (2.3–6.4)	1.59 (0.6–2.8)	<0.001*
ANG1 (ng/ml)	61.5 (34.3–94.9)	29.3 (19.4–45.6)	<0.001*
ANG1/ANG2 ratio	13.8 (9.0–27.6)	24.1 (9.7–53.1)	0.11
sTIE2 (ng/ml)	52.8 (37.3–119.4)	24.5 (21.4–34.4)	<0.001*
PROK 2 (ng/ml)	28.6 (28.2–29.7)	28.1 (28.0–28.5)	<0.001*
PROK 1 (ng/ml)	335.9 (335.5–340.8)	335.7 (335.4–336.8)	0.40

Table 6. Angiogenic markers in patients and controls. Values are expressed as median (25th–75th percentile) Comparisons have been performed by Mann–Whitney test. NEN: neuroendocrine neoplasm, ANG: angiopoietin, PROK: prokineticin, sTIE2 soluble TIE2. *Statistically significant difference between groups.

4.2 Angiogenic markers in NENs patients: morphology and staging

We, therefore, analyzed angiogenic markers within NENs patients to assess any difference according to tumor morphology and staging. ANG2 levels were significantly higher in NECs (4.9 ng/ml, 2.8–7.4) compared to NETs (3.2 ng/ml, 1.7–6.4), p=0.046. Evaluating patients according to staging (TNM stage 1 and 2 vs TNM stage 3 and 4), ANG2 and sTIE2 were both higher in locally advanced or metastatic diseases (TNM 3-4): ANG2: 4.2 (2.7–8.7) vs 2.7 (1.5–5.7) ng/ml, p = 0.044, and sTIE2: 67.4 (44.7–142.0) vs 46.5 (22.3–103.7) ng/ml, p = 0.032. No difference was found in other angiogenic markers; data are summarized in Figure 4 and Table 7.

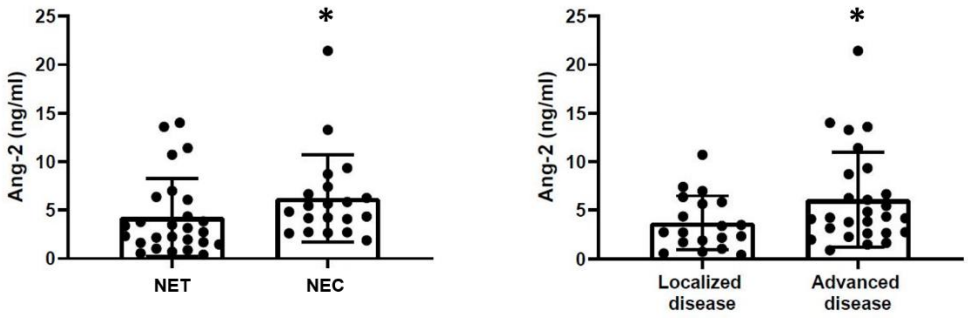

Figure 4. Circulating angiogenic factor levels in patients affected by NENs, according to morphology and stage. Values are reported as mean ± standard error of mean. NEN: neuroendocrine neoplasms, ANG: angiopoietin, NET: neuroendocrine tumor, NEC: neuroendocrine carcinoma.

	Morphology (well vs poorly differentiated)			Staging (TNM 1-2 vs 3-4)		
	Well differentiated	**Poorly differentiated**	**P**	**TNM 1-2**	**TNM 3-4**	**p**
ANG2 (ng/ml)	3.2 (1.7–6.3)	4.9 (2.8–7.4)	0.046*	2.7 (1.5–5.7)	4.2 (2.7–8.7)	0.044*
ANG1 (ng/ml)	58.0 (32.8–91.8)	83.7 (43.6–104.1)	0.746	71.0 (34.8–91.8)	59.6 (32.8–104.1)	0.982
ANG1/ANG2 ratio	17.3 (9.0–73.5)	11.7 (9.0–24.2)	0.378	18.7 (10.4–33.8)	10.7 (6.3–24.2)	0.116
sTIE2 (ng/ml)	49.3 (29.3–109.3)	58.6 (37.7–142.0)	0.310	46.5 (22.3–103.7)	67.4 (44.6–142.0)	0.032
PROK2 (ng/ml)	28.5 (28.2–29.5)	28.7 (28.3–32.6)	0.235	28.5 (28.2–29.4)	28.7 (28.3–30.1)	0.502
PROK1 (ng/ml)	337.3 (335.4–343.9)	335.5 (335.5–335.9)	0.117	335.6 (335.4–342.5)	335.9 (335.5–356.7)	0.505

Table 7. Angiogenic markers in NENs patients: morphology and staging. Values are expressed as median (25th–75th percentile). Comparisons have been performed by Mann–Whitney test. ANG angiopoietin, PROK prokineticin, sTIE2 soluble TIE2. *Statistically significance difference between groups.

4.3 Angiogenic markers in NENs patients: disease status

We first assessed the difference in angiogenic markers according to disease status (stable or progressive disease) at the moment of blood sampling, as reported in Figure 5 and Table 8. ANG2 levels were significantly higher in patients with progressive disease (6.26 ng/ml, 3.98–10.99) than in patients with stable disease (2.73 ng/ml, 1.65–4.36, p = 0.001) while ANG1/ANG2 ratio was significantly lower 10.23 (4.60–12.90) compared to 21.55 (10.35–66.83), p = 0.002.

PROK2 levels were higher in patients with progressive disease, 29.19 pg/ml (28.42–32.25) compared to patients with stable disease 28.37 pg/ml (28.14–28.91), p = 0.035.

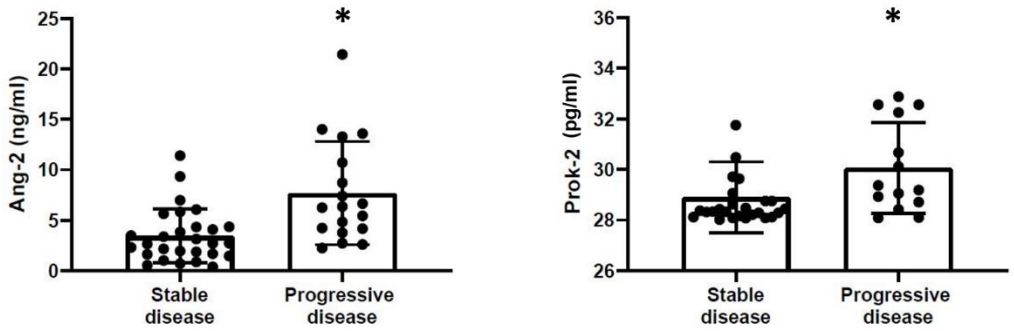

Figure 5. Circulating angiogenic factor levels in patients affected by NENs, according to disease status (stable vs progressive disease). Values are reported as mean ± standard error of the mean. ANG: angiopoietin, PROK: prokineticin.

	Progression (at the moment of sampling)			Progression (1 year of follow-up)		
	Stable Disease	**Progressive disease**	**p**	**Stable disease**	**Progressive disease/death**	**p**
ANG2 (ng/ml)	2.7 (1.6–4.4)	6.3 (4.0–11.0)	0.001*	2.3 (1.5-3.8)	6.3 (4.2-10.1)	<0.001*
ANG1 (ng/ml)	71.8 (47.4–91.8)	54.6 (30.2–105.2)	0.471	58.0 (4.8-91.8)	59.6 (32.0-91.8)	0.748
ANG1/ANG2 ratio	21.6 (10.4–66.8)	10.2 (4.6–12.9)	0.002*	21.2 (11.3-73.5)	10.4 (4.7-15.7)	0.007*
sTIE2 (ng/ml)	49.1 (35.9–108.2)	76.7 (42.9–145.5)	0.150	50.7 (29.3-108.8)	85.9 (39.8-147.3)	0.156
PROK2 (ng/ml)	28.4 (28.1–28.9)	29.2 (28.4–32.3)	0.035*	28.5 (28.1-29.1)	28.6 (28.2-30.4)	0.703
PROK1 (ng/ml)	335.8 (335.4–343.1)	335.9 (335.5–338.1)	0.962	336.5 (35.4-344.9)	335.6 (335.5-336.7)	0.294

Table 8. Angiogenic markers in NENs patients according to progression at baseline and after follow-up (1 year). Values are expressed as median (25th–75th percentile). Comparisons have been performed by Mann–Whitney test. ANG angiopoietin, PROK prokineticin, sTIE2 soluble TIE2. *Statistically significance difference between groups.

ROC analysis revealed an AUC of 0.81 (95% confidence interval: 0.68–0.93, $p < 0.001$) for ANG2 in differentiating patients with stable or progressive disease, and a cut-off of 3.5 ng/ml demonstrated a sensibility of 84% and specificity of 65% in identifying progressive disease (Figure 6).

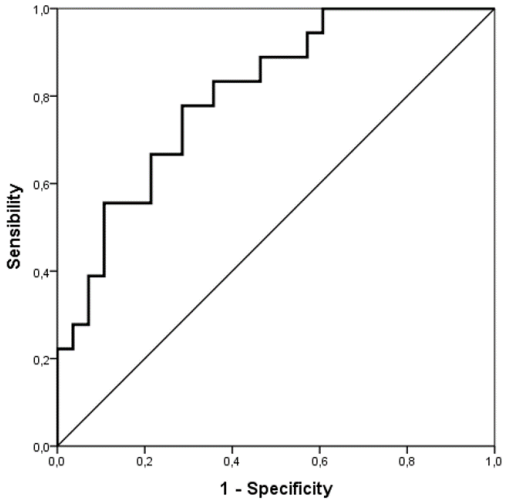

Figure 6. ROC curve for Angiopoietin 2 (AUC 0.81).

We also evaluated if the levels of angiogenic markers could predict progression (or death) in the following 12 months of follow-up: data confirmed that ANG2 was higher in patients with progression during follow-up compared with patients with stable disease: 2.3 (1.5-3.8) ng/ml *vs* 6.3 (4.2-10.1) ng/ml, p<0.001. Accordingly, ANG1/ANG2 ratio was significantly lower in patients with progressive disease, as reported in Table 8.

4.4 Immunohistochemistry

Tissue specimen was available in a subgroup of 10 patients (eight GEP-NENs and two lung NENs). In all cases, immunohistochemistry confirmed the expression of ANG2 and ANG1 in tumor specimens. The double staining imaging (for ANG2 and chromogranin) highlighted the expression of ANG2 in both NETs (with high expression of chromogranin) and NEC (characterized by lower expression of chromogranin).

Immunohistochemistry also demonstrated, for the first time, the expression of PROK2 in the tumor and the stromal tissue, as reported in Figure 7.

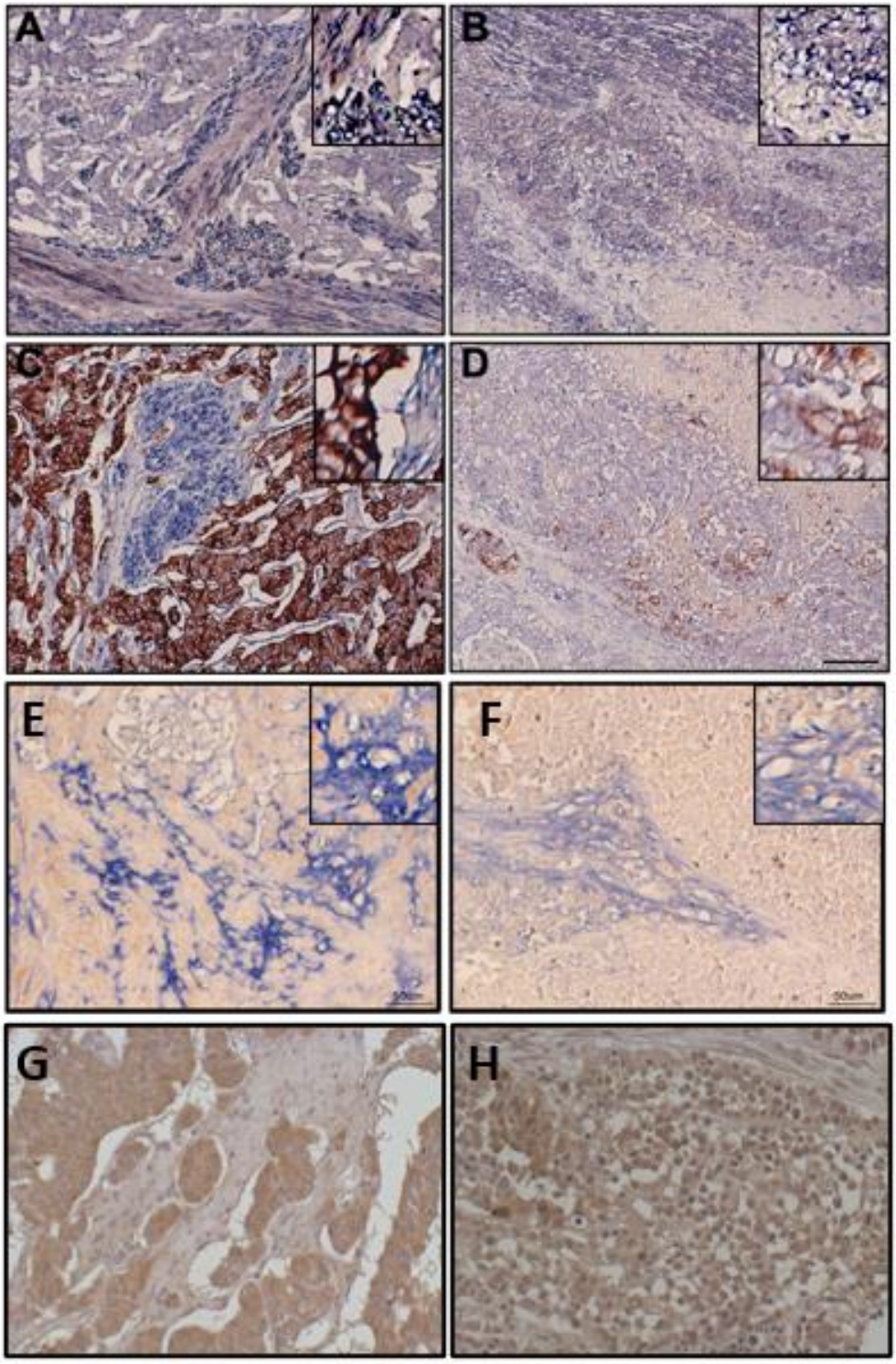

Figure 7. Analysis of Angiopoietins expression in FFPE sections from grade 1 GEP-NET (A, C, E, G) and grade 3 GEP-NEC (B, D, F, H) tissues. Sections were single-stained with ANG2 antibody (A-B), double-stained with ANG2 + Chromogranin A (C-D), single-stained with ANG1 antibodies (E-F), and single-stained with PROK2 (G-H). Positive staining for ANG2 and ANG1 is indicated by the blu AP signal, Chromogranin A staining is indicated by the brown DAB signal, and PROK2 staining is indicated by the brown signal. Scale bar = 100 μm; E-F = 50μm. Inset magnification = 4X.

4.5 Sequencing of circRNA from TEPs: preliminary findings

Ten patients from the main study have been enrolled also in the pilot proof of concept study. All patients were affected by GEP-NETS (6 pancreatic, 4 small intestinal). 8 had metastatic disease at diagnosis, and 2 had localized disease (T1N0). The mean age was 60.9 ± 9.1 years (3 females and 7 males). All patients were naïve to any treatment at the moment of sampling. For a subgroup of 5 patients, a follow-up evaluation was available (3 months after treatment). Table 9 summarized the characteristics of the patients.

	NENs (10)	**CONTROLS (5)**	**p VALUE**
Age (years)	60.9 ± 9.1	58.8 ± 10.9	0.698
Sex (m/f, n)	7/3	2/3	0.264
Smoking habits	0 (0%)	0 (0%)	NA
Arterial hypertension	4 (40%)	2 (40%)	1.000
Diabetes mellitus	1 (10%)	1 (20%)	1.000
Primary sites	6 pancreas 4 small intestine	-	-
TNM stage	2 localized 8 metastatic	-	-
Grade	4 grade 1 6 grade 2	-	-

Preliminary results demonstrated a large number of circRNA found in this study, equal to 98,735, of which 63,562 were not previously annotated and 35,173 annotated in circBase database[122] as reported in Figure 8.

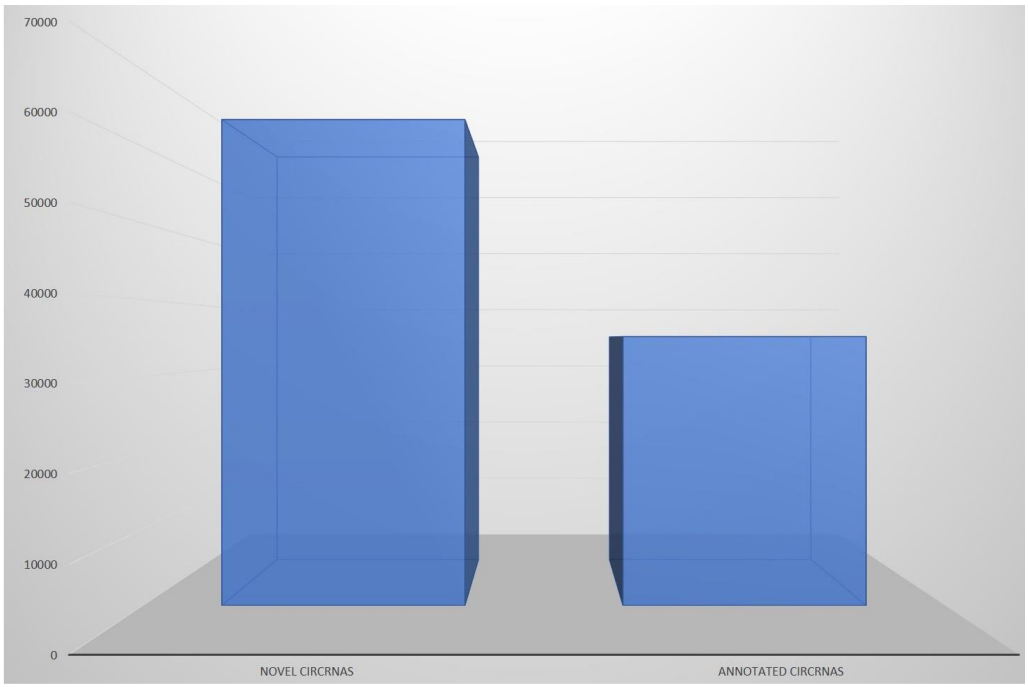

Figure 8. Novel and annotated circRNA found in this study.

The average number of annotated circRNA for chromosomes is reported in Figure 9.

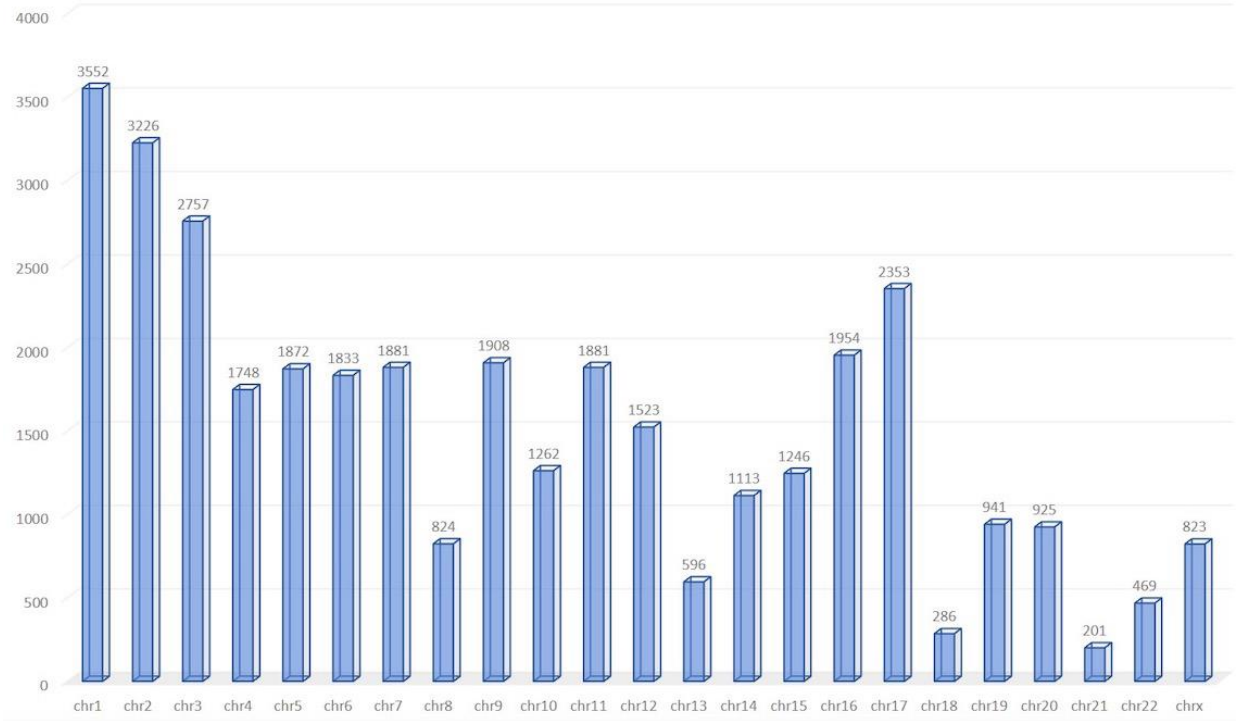

Figure 9. The number of annotated circRNA per human chromosome. Chr: chromosome.

A bioinformatic analysis is ongoing to evaluate differently expressed circRNA from TEPs in patients and controls, to investigate if circRNAs from TEPs could be used as diagnostic

biomarkers for GEP-NET. In addition, from the analysis of differentially expressed circRNA in patients before and after treatment in a subgroup of the same NET patients, we will explore the potential early prognostic role of circRNAs expression profile.

5. DISCUSSION

The availability of circulating biomarkers for the detection and characterization of NENs is one of the unmet needs in the neuroendocrine field[57]. The diagnosis can be challenging, especially for non-functioning NENs, and can have a marked delay[21]. In addition, tumors with similar morphological characteristics and stages can show different behavior and response to treatment, making necessary the research of prognostic and predictive markers[125]. In this study, we evaluated the role of angiogenetic markers and the expression profile of circRNA from TEPs in NENs.

One of the peculiar features of NENs is the high vessel density. Nevertheless, studies analyzing the circulating angiogenic markers in NENs are scanty and sometimes contrasting. In this prospective study, we demonstrated statistically significant higher levels of ANG2 and ANG1 in patients affected by NENs compared to controls, similar to some previous studies. The study with the highest number of patients enrolled (90 patients compared to 40 healthy controls), conducted by Detien *et al.*, demonstrated that circulating levels of ANG2 are higher in patients affected by GEP NEN[112]. Similar findings were derived from two other studies on 42[124] and 47 patients[126] with GEP-NENs. On the contrary, another study demonstrates no differences in ANG2 levels between patients and controls (36 enrolled, both pulmonary and GEP-NENs)[127]. Data on ANG1 levels are available in 3 studies; one revealed an increase of this angiogenic marker in patients compared to controls[124], while the other two studies showed no difference[126,127]. ANGs act by binding to their receptor, Tie2. Its soluble portion, sTie2, derives from the proteolytical cleavage of the receptor on the cell surface and can be measured in serum. Our study demonstrated an increase in sTie2 in patients affected by NENs, according to other two studies[124,127]. Overall, patients affected by NENs showed higher levels of ANGs and sTie2 than controls.

We, therefore, tried to analyze the levels of angiogenic markers with respect to neoplastic characteristics, within the group of NENs patients. Regarding TNM staging, our results demonstrated higher levels of ANG2 and sTIE2 in locally advanced or metastatic disease compared to localized disease. Accordingly, other studies reported that ANG2 serum levels

were higher in metastatic tumor[112,124,126,127]. Pre-clinical data on NET-xenograft clarified that nodal diffusion of GEP-NET was more frequent in ANG2-expressing tumor[112] and clinical studies revealed no difference in angiogenic factors according to the primary site of the tumor[112,127]. These data could be explained by the fact that angiogenesis is more implicated in tumor growth and metastatization of NENs independently from the primary tumor site.

Our study was the first to investigate angiogenic markers according to grade and morphology (well and poorly differentiated NENs). ANG2 levels were significantly higher in patients affected by NECs, suggesting that ANG2 could be able to identify more aggressive forms. The possible prognostic role of this marker has been corroborated by the findings that ANG2 levels were higher in patients with progressive diseases, regardless of the standard therapy used (SSA for well-differentiated tumors and chemotherapy with cisplatin and etoposide in NEC). In addition, circulating levels of ANG1 were not higher in patients with tumor progression, and coherently ANG1/ANG2 ratio was lower. This confirmed the angiogenetic switch, with ANG2 acting as a Tie2 antagonist, leading to angiogenesis and vascular instability, therefore promoting tumor growth[110,128]. In addition, ANG2 was also higher in patients who experienced tumor progression in the 12 months after enrollment in the study, similar to another study that demonstrated a shorter time to disease progression in NENs patients with higher ANG2 levels[126]. Our results strengthen the evidence of the possible role of ANG2 circulating levels as a prognostic marker. This is of clinical significance since the accuracy of available circulating prognostic markers is poor.

This was the first study measuring levels of PROK1 and 2 in NENs, expanding the panel of the angiogenic factors evaluated in NENs. We found that PROK2 levels were higher in NENs than in controls, without difference comparing patients for tumor stage (localized vs locally advanced or metastatic) and tumor morphology (well-differentiated vs poorly differentiated forms). Remarkably, PROK2 demonstrated a prognostic role: its levels were higher in patients with disease progression at study entry. This data suggest the potential role of PROKs in NENs development, therefore future studies are needed both to improve the knowledge of NENs biology and of the possible role of PROKs as circulating biomarkers.

The demonstration by immunohistochemistry of the presence of both PROK1 and ANG2 in tumor tissues validated the hypothesis that high circulating levels of angiogenic markers originated from tumor cells.

As a possible future perspective, a deeper understanding of the angiogenic pathway involved in NENs progression and metastatization could be of great importance also for treatment. Recent clinical trials evaluated the efficacy of ANG inhibitors, trebanib, rebastinib, and MEDI3617 in many tumors, such as ovarian cancers, endometrial cancer, breast cancer, and gastrointestinal malignancies[129]. In murine models of pancreatic NETs, ANG inhibitors were able to overcome VEGF resistance, suppressing revascularization and tumor progression[130].

In the pilot study, we performed a sequencing analysis of circRNA from TEPs of patients affected by well-differentiated NETs. It has been demonstrated that the RNA content of circulating platelets is modified by cancer, therefore providing the opportunity to use circRNAs from platelets as diagnostic, prognostic, and predictive biomarkers[93]. This approach has been used in many kinds of cancers, including non-small cell lung cancer[131], endometrial cancer[132], colorectal cancer[133], glioblastoma[134], renal cell carcinoma[135], sarcoma[136], and hepatocellular carcinoma[137] but no data are available in NENs. We sequenced up to one hundred thousand circRNA expressed in patients with NETs and controls, with most of them not previously annotated in databases[122]. This data confirmed the potential validity of this strategy also in well-differentiated NETs, differently from other sources of liquid biopsy, such as ctDNA, which are less released in the case of well-differentiated NENs. Bioinformatic analyses are currently ongoing for exploring the role of circRNA profiling as diagnostic biomarkers of NETs (from the comparison of circRNA differentially expressed in patients and controls) and as an early prognostic and predictive role (comparing circRNA expression profile in the same patients before and after treatment). The study will lay the foundation for the use of this kind of liquid biopsy both for diagnostic and prognostic purposes in NETs.

The main limitation of the main study is that most of the patients enrolled have been previously treated by medical therapy (SSA for well-differentiated tumors and cisplatin-etoposide for poorly differentiated carcinomas). Therefore, it is not possible to exclude an interference on angiogenic markers circulating levels, since treatment-induced modification in the tumor microenvironment, such as inflammation and hypoxia, could increase the level of angiogenic factors[128]. For the pilot study, given the low number of samples analyzed, it will be not possible to draw a definitive conclusion on the diagnostic and prognostic role of circRNA sequencing from TEPs.

6. CONCLUSIONS

This study substantiated the evidence of the role of angiogenic markers in NENs. ANG2, ANG1, sTIE2, and PROK2 were all higher in patients with NENs compared with controls. The circulating levels of these markers, in particular ANG2, were correlated with tumor stage and grade. Remarkably, both PROK2 and ANG2 demonstrated a correlation with tumor progression, suggesting their role as prognostic circulating biomarkers, which are an unmet need in Neuroendocrine fields. Our study also demonstrated that TEPs are a good source of circRNA in patients affected by NENs, unrevealing also a great amount of circRNA not previously annotated. In this context, the data coming from the sequencing of circRNAs from TEPs in NEN could be the base for the development of novel markers for the diagnosis and follow-up of patients but also for deepening the knowledge of NENs biology. Revealing the pathways overexpressed or suppressed in cancers could potentially guide treatment choice, including combination treatment, through the prediction of treatment response and a better understanding of drug resistance.

7. REFERENCES

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