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Review Article

NETest: a systematic review focusing on the prognostic and predictive role

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Short Title: Prognostic and predictive role of NETest

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Abstract
The NETest is a standardized and reproducible liquid biopsy for neuroendocrine tumors (NETs). It evaluates the expression of 51 NET genes by real time polymerase chain reaction, providing an accurate molecular profile of the neoplasm. Diagnostic utility of NETest has been widely demonstrated, while its role in predicting prognosis and treatment response is less studied. This systematic review aims to collect and discuss the available evidence on the prognostic and predictive role of NETest, trying to answer 3 questions, frequently raised in clinical practice. *Is NETest able to differentiate stable from progressive disease?* Increased NETest levels (at least >40%) correlate with disease progression. *Is NETest able to predict tumor progression and tumor response to treatment?* Some studies demonstrated that baseline NETest score higher than 33-40% could predict tumor progression. Moreover, NETest performed after treatment (as PRRT) could predict treatment response also before radiological findings, since the decrease or stability of NETest score predicts tumor response to treatment. *Is NETest able to evaluate tumor recurrence risk after surgery?* NETest can predict surgical treatment outcome detecting minimal residual disease after radical surgery, which is characterized by lower but positive NETest score (20-40%), while higher score (>33-40%) is associated with non-radical surgery.
In conclusion, in addition to its demonstrated diagnostic role, this systematic review highlights the efficacy of NETest to assess disease status at the moment of the NETest execution and to predict tumor recurrence after surgery. The efficacy for other applications should be proven by additional studies.

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Introduction

Neuroendocrine neoplasms (NENs) are heterogeneous in term of primary sites, neuroendocrine differentiation, clinical behavior, and response to treatments. In this field, the possibility to relay on easy to execute markers for estimating the prognosis and for predicting response to treatment could be essential for improving the clinical management of these patients [1]. Nowadays in patients affected by neuroendocrine tumors the strongest predictors of overall survival are tumor grade and stage [2].

Circulating biomarkers have been studied for diagnosis and follow-up of patients affected by NENs. While in functional NENs it is possible to analyze secretory products or their metabolites in blood and/or in urinary sample, in non-functioning tumors only the so-called “general tumor markers” can be used [3]. Chromogranin A (CgA) is the most used general tumor marker, both with diagnostic and predictive value [4]. The prognostic role of this marker has been advocated since the demonstration of the correlation with overall survival [5], poorer outcome [6] and tumor burden [7]. However, there are multiple limitation of CgA use. False positive value can be observed in non-oncological diseases, such as atrophic gastritis, hypergastrinemia, heterophile antibodies, impaired kidney function or use of anti-secretory medications, especially proton pump inhibitors [8, 3, 9]. False negative results can happen in case of less differentiated disease, since CgA is a secretory product of the neuroendocrine cells [10].

Neuron-specific enolase (NSE) is physiologically present in neurons and neuroendocrine cell and its circulating form is a biomarker for overall survival in gastro-entero-pancreatic (GEP) NENs patients [11, 12]. However, serum NSE is increased only in 30-50% of these patients [13]. A significant association of NSE levels with survival was also demonstrated in patients affected by small cell lung cancer (SCLC) [14]. As a prognostic marker of neuroendocrine tumors, NSE is minimally correlated with tumor size but associated with grading. In fact, its levels result higher in patients with poorly differentiated NEC [15].

Preliminary data of the use of serum neutrophil-lymphocyte ratio (NLR) are available in patients affected by lung neuroendocrine carcinomas. In these patients an increasing preoperative NLR is associated with higher stage and inversely correlates with post-resection overall survival (OS) and relapse free survival (RFS) [16, 17]. The predictive value of worse survival of the preoperative NLR was also demonstrated in patients with gastric NENs undergoing surgery [18] and in intestinal and pancreatic NETs (Ki-67 < 10%) treated with lanreotide [19].

Recently, NETest has been developed and proposed mostly for the diagnosis of NENs, demonstrating in a recent metanalysis on 10 studies a diagnostic accuracy of 95% - 96% [20].

The aim of this systematic review is to collect and discuss the available evidence on the role of NETest in predicting prognosis and treatment response, including both systemic treatments used in metastatic patients and surgical and ablative strategies used in localized disease, in patients affected by NENs, trying to answer the following questions: 1) Is NETest able to differentiate stable from progressive disease? 2) Is NETest able to predict tumor progression and response to therapy? 3) Is NETest able to predict tumor recurrence after surgery?

Materials and Methods

We performed this study according to the Cochrane Collaboration and PRISMA statement [21].

Data sources and searches

From June to November 2020 we searched for English-language articles in MEDLINE. No date restriction has been applied. Search terms used were: “NETest”; “predictive biomarker” AND “neuroendocrine”; “prognostic biomarker” AND “neuroendocrine”; "liquid biopsy" AND "neuroendocrine". Additionally, we searched in EMBASE, Cochrane Library and SCOPUS using “NETest” as search term.

Eligibility criteria for study selection included studies on humans with any of the following design: randomized clinical trials, prospective non-randomized trials, retrospective studies and case series.

We selected: 1) article on NETest; 2) data on prognostic value or treatment response prediction or treatment response assessment of NETest 3) patients affected by any subtypes of NENs.

Exclusion criteria were: 1) non original articles or case reports; 2) article reporting only data on the diagnostic value of NETest. A final update of the search was conducted in May 2021 and one additional study was included.

Article Selection

Each study was screened by abstract and title and potentially eligible studies were further assessed in detail by retrieving full-length articles. Each full-length article was independently reviewed by two separate Authors (GP

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and VDV) following inclusion criteria. Two authors independently extracted data from the articles that met the inclusion criteria. A standardized form was used to extract the following information: year of publication, type of study, number of included patients, age at diagnosis, sex, histopathological examination, staging and outcomes, treatment strategy (surgery, medical treatment, radiotherapy), time of execution and values of NETest, correlation of NETest with disease status, prognosis and treatment response. Quality of studies has been assessed by MINORS score [22].

Results

From the original number of 244 articles, 26 have been selected by title and abstract. After full-text evaluation, a total of 20 articles were included in the systematic review (Figure 1).

Liquid biopsy and NETest

In recent years liquid biopsy has received growing attention. It is a molecular biology technique that allows to identify and to characterize neoplasms, through molecular analyses performed on a venous blood sample. Liquid biopsy is performed analyzing the following elements: circulating cancer cells, free nucleic acids, exosomes and Tumor Educated Platelets (TEPs); the name of the latter originates from their ability to engulf the circulating RNA released by the tumor, with therefore a correlation between TEPs and tumor growth and dissemination in various kind of neoplasms [23, 24]. One of the advantages of liquid biopsy analysis is that it can be serially repeated, allowing to get real time information from the lesion so that we can promptly make changes in therapy [25]; moreover, given the characteristic heterogeneity of neuroendocrine neoplasia, this diagnostic strategy could probably be more effective in representing the totality of the disease compared to a biopsy that could only provide a partial view [26, 27].

In this field of liquid biopsy applied to NENs, NETest has been developed in recent years. This is a standardized and reproducible clinical laboratory measurement for the diagnosis of NENs, whose clinical utility has been documented in GEP and bronchopulmonary NENs and in paragangliomas and pheochromocytomas (PPGLs) [28-31]. The efficacy of this biomarker for diagnostic purpose originates also from its independence from patient's characteristics, such as age, sex or ethnicity, and treatments. As a result, it is advantageous compared to CgA [31, 32].

After mRNA isolation and cDNA production, NETest uses Real Time Polymerase Chain Reaction (RT-PCR) in order to quantify circulating transcriptional products of neoplastic origin. In particular, NETest evaluates the expression of 51 genes, 30 of which can be categorized into nine clusters, related to cell proliferation and apoptosis (Proliferome, Growth factor signalome, Apoptoma), peptide secretion (Secretome I, general and Secretome II, progressive) epigenomic changes (Epigenome) and somatostatin receptor (SSTRome), providing tumor molecular profile [33, 34]. After RT-PCR, results are analyzed through algorithms, designed to differentiate healthy controls from NENs patients, determining a 0-8 score: samples scored 0 - 2 are classified as normal, while levels 3 - 8 are categorized as NENs [35]. To expand the utility of the NETest from a diagnostic tool to an instrument able to capture the biology of NENs, a second algorithm-based analysis quantifies the expression of 6 of the above-mentioned clusters (SSTRome, Proliferome, Metaboloma, Secretoma, Epigenome, Pluroma) and, incorporated also the machine-learning derived 0–8 score, generates a clinical activity score scaled from 0 to 100% (the NETest score). While the first score is able to diagnose NENs from controls, the second score is related to tumor activity, being more able to differentiate stable from progressive disease [31]. Thus, the NETest is able to capture the biology of a specific NEN defining its molecular status.

Prognostic role of NETest

Is NETest able to differentiate stable from progressive disease?

Ten studies have demonstrated that NETest is able to differentiate between stable (SD) and progressive diseases (PD), in many subtypes of NENs (Table 1).

Using the initial linear score, ranging for 0 to 8, Kidd *et al.* demonstrated that a score between 0 and 5 was associated with stable disease in over 90% of patients while a score of 8 correctly identified progressive disease in over 90% of patients. The study was performed on an overall population of 111 patients with SD and 48 with PD, mostly grade 1-2 GEP-NET [34]. In the same study, using the NETest score, the authors demonstrated that SD

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samples had significantly lower activity scores than PD samples (34.1±27% vs 83.7±24.4%, p<0.0001), proposing 45% as a cut-off (upper 95% confidence interval for SD) [34].
Malkzwesca *et al*., in a study on 72 patients affected by radiologically detectable GEP-NENs (42 PNET, 33 SINET), 11 progressive and 64 stable, confirmed that NETest score was significantly increased in PD (mean ± SD: 61 ± 26%) compared to SD (29 ± 14)%; the cut-off score of 40% showed an accuracy of 95% [36]. Similar results were also confirmed by Pavel *et al*. (PD group vs SD group 67.2 ± 7.1 % vs. 41.6 ± 5.8 %, p < 0.05) [37].
The ability of NETest to differentiated PD from SD was confirmed also in studies enrolling only pulmonary NENs. In a study on 112 patients, NETest score was significantly lower in SD (36 ± 19%) than in PD (73 ± 22%, p < 0.001) [38]. In a cohort of 25 typical and atypical lung carcinoids, NETest was confirmed to differentiate tumor progression (85 ± 11% vs 32 ± 7%) [30]. Despite the good performance, as demonstrated by high AUC, in both cases the authors did not propose a specific cut-off [38, 30]. Another study confirmed that NETest accurately distinguished PD from SD (61 ± 26 *vs* 35.5 ± 18; p <0.0001); moreover, in pulmonary carcinoids (data on 99 patients), NETest levels were elevated in metastatic disease irrespective of histology (typical, 78 patients, and atypical carcinoids, 21 patients), both in SD cohort than in PD cohort [39].
A recent study by Malczewskaa *et al.* confirmed, on a large series of patients (135 GEP and 64 pulmonary), that NETest was able to differentiate PD from SD both in GEP-NENs (55±5 *vs* 34±2, p=0.0005) and in pulmonary carcinoids, (57.8±7 *vs* 29.4 ±1 p<0.0001) [40] and PPGLs [29].
On the other hand, other articles identified a cut-off of 80% for PD [41]. This higher cut-off (80%) has been identified also in the study by Liu *et al.* The study demonstrated the high concordance between low NETest (<40%) and SD (83% of patients with SD) and high NETest (>80%) and PD (60% of patients with PD), with an overall concordance of 88% [42].
Other studies demonstrated that a significant difference in NETest score is also found between localized and metastatic disease, both in GEP and pulmonary NENs [43, 40].
A study on patients affected by PPGLs identified a cut-off of 53% for differentiating PD (11 patients) and SD (19 patients) at the moment of the blood collection (0.93, CI: 0.84– 1.03), p<0.0001); moreover, NETest score was significantly higher in multicentric and metastatic disease than in localized disease [29].
In summary, NETest score higher than 40% demonstrated a high concordance with progression evidenced by radiological imaging. This finding does not change also considering prospective studies only. This is in accordance with the cut-off of 40% reported in the metanalysis by Oberg et al [20].

Is NETest able to predict tumor progression and tumor response to treatment?
Some studies have evaluated the possibility for NETest to predict tumor progression in the months following the execution of the NETest (Table 2).
An interesting study by Pavel *et al*, on 34 patients affected by stable GEP-NETs, demonstrated that, in patients with stable disease at baseline, the basal value of NETest higher than 40% can predict subsequent tumor progression despite multimodal treatment strategies, while values lower than 40% predicts stability over 5 years. Moreover, basal NETest score was associated with progression free survival (PFS) (hazard ratio = 1.022, 95% confidence interval = 1.005–1.04; p < 0.012) and Kaplan-Meier analyses demonstrated that baseline NETest lower than 40% predicted longer PFS (median PFS 2.78 years, in case of basal NETest <40% and 0.68 years in case of basal NETest >80%) [37].
The correlation of basal NETest score with PFS was confirmed also in a cohort of 100 patients (68 GEP, 20 BP and 12 mixed NENs). Mean PFS of patients with NETest scores lower than 40% without treatment was 12 months, while mean PFS was only 3 months in case of basal NETest scores higher than 80, despite treatment [42]. In the same study, Liu *et al.*, evaluated the utility of the NETest in a watch-and-wait program (45 patients). Patients with a basal low score (≤40%; n = 27) maintained stable disease, while all patients with a high NETest (≥80%; n = 14) required treatment intervention and/or developed PD [42].
A recent interesting prospective study has enrolled 152 patients with sporadic GEP-NENs, followed for 36 months (range 4–56). 119 had measurable disease and 33 had no evidence of disease at enrolling. Basal NETest categories (low tumor activity <33%; intermediate tumor activity: 34–79%, and high tumor activity ≥ 80%) predict median PFS, which was respectively 55, 18 and 11 months. Patients with NETest > 33% had an overall 9 times higher risk of developing PD compared to those with NETest ≤33% (with a reported odds ratio of 8.6). Of the overall number of 152 patients, 55 with measurable disease were enrolled in the watchful waiting group (no treatment) and 32% of these patients developed PD within 1 year. Only 16% of patients with low tumor activity had PD, compared to

50% and 54% of intermediate and high activity categories. In parallel, 64 patients were treated since baseline and PD within 12 months of follow-up was observed in 45%. Once again, basal NETest predicted the risk of PD at 12 months: progression was observed in 17% of patients with low activity scores, in 61% with intermediate and in 74% with high tumor activity. As confirmation, 70% of patients with low tumor activity in the watchful waiting group and 64% of patients with low tumor activity in the treatment group had SD after 24 months. Finally, considering the 33 patients without evidence of disease at baseline, no patients with negative NETest (<20%) developed recurrence; moreover the median value of NETest in patients who remain free of disease at follow-up was 27% compared to 53% in patients with recurrence [44].
Cwikla *et al.*, in the prospective part of their study, evaluated NETest score before starting somatostatin analogs (SSA) treatment for GEP-NETs (G1 and G2). A basal level of 80% predict the development of disease progression in 100% of patients (14 patients). Even in case of basal NETest under 80%, NETest score was higher in patients (57.5 ± 6% vs 41± 2%; p=0.02) that subsequently developed PD [41].
Some studies, reporting multiple NETest determinations, evaluated if NETest score variation could assess treatment efficacy. These studies focused mainly on Peptide Receptor Radionuclide Therapy (PRRT) and SSA. Bodei *et al*. evaluated the changing in NETest score according to PRRT efficacy. In this study, responders to PRRT were defined as patients showing complete response, partial response or stable disease evaluated by computed tomography according to Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1, while non responders showed tumor progression. After 6 months from treatment starting, a reduction in NETest was seen in 88% of responder patients (no change in 12% of responders) while an increase in NETest was seen in 90% of non-responders, showing high agreement between radiological findings and NETest (89 % of concordance) [45]. These preliminary results have been subsequently confirmed by another study on 122 patients affected by lung and GEP-NETs. In case of objective response to PRRT NETest decreased (mean change: -47 ± 3%), while NETest remains high in the majority of non-responders. Authors proposed a post-treatment cut-off of 40% for differentiating SD from PD and therefore for determining PRRT response [46].
In a study on repeated NETest measurements on 9 patients affected by PPGLs, 4 patients with SD at baseline and a good response to treatment with SSA demonstrated a fall or a stability in NETest score at follow-up, while 2 SD at baseline with following PD showed an increase in NETest score. Considering patients with PD at baseline, NETest score increases in 1 patient with further progression, decrease in 1 patient who underwent surgery and remains stable in 1 patient without further progression on SSA treatment [29].
Cwikla *et al.* demonstrated that elevated NETest (80–100%) during SSA treatment for GEP-NETs (G1 and G2) was significantly associated with therapy failure (sensitivity, 100%; specificity, 57%; PPV, 70%; and NPV, 100%)[41].
Liu et al, in a cohort of 55 NENs patients treated mainly with SSA (84%), confirmed that NETest was clinically useful in treatment monitoring. In fact, 100% of patients with a low score exhibited stable disease at 6–12 months, while an increase in NETest during treatment required further therapy modifications, as increase in SSA dosage or addition of an alternative agent [42].
Overall, high NETest values (>40%) in patients without contemporary radiological progression could predict subsequent progression. All the available studies are prospective, but since study treatments and endpoints vary between studies, no definitive conclusions on the application of NETest in this clinical context can be deduced.

Is NETest able to evaluate tumor recurrence risk after surgery?
In a study on 35 GEP-NENs (only 1 G3), in which 27 patients underwent surgical resection and 8 embolization [35], resection was associated to a decrease in NETest score (from 80 ± 5% to 29% ± 5, p <0.0001). The study included NETest evaluation 1 month after treatment and radiological evaluation 3 and 6 months after treatment. Interestingly, considering the surgical cohort, authors compared 15 R0 patients with 12 R1 patients. In the group with radical surgical resection the reduction in NETest score was more marked (R0: from 80 ± 6.3% to 28.9 ± 5.5%; R1: 79.5 ± 8.5% 47.2 ± 9.9%). Moreover, the same authors reported that 12 patients with R0 and no clinical or radiologic evidence of disease recurrence after 5 years showed a maximum post-operative NETest score of 14% (negative score), while in other cases NETest score showed a moderate disease activity (>20%). This is consistent with the finding that 4 of 11 R0 patients with increased NETest at 1 month developed subsequent positive imaging (sensitivity 100%, specificity 20%) [35].
In a study on 13 small intestine NETs, surgery determined a significant lowering of NETest score. However, a significant reduction was associated to curative surgery (NETest 20-40%, classified as low) while, in case of

resection in the setting of metastatic disease the reduction was lower; in particular in case of progressive disease after surgery, NETest score was above the levels of 40% (over 60% in 3/5 cases) [47].
Similarly, in a study reporting NETest score before and 6 months after a combined treatment with both surgical intervention and PRRT in 9 patients affected by metastatic small intestinal NETs, authors reported a reduction in NETest score after treatment (from 83 ± 12 % to 34 ± 15%) and identified 40% as post-treatment cut-off for detecting stable disease [48].
Genc *et al.* analyzed the role of NETest, performed after surgery, in predicting recurrence of pancreatic NETs (G1 and G2), identifying a cut-off of 40% (false positive or false negative patients were 18%). Interestingly, NETest was higher in patients with recurrence (R0 at histology) that in patients diagnosed as R1 but without clinical recurrence [49].
Partelli *et al*. evaluated NETest before and after surgery on 30 patients affected by pancreatic NENs. Beyond the efficacy of NETest in diagnosing NETs before surgery, after surgery all patients demonstrated a decrease in NETest levels without differences in each time evaluated (post-operative day, POD 1, 5 and 30). Interestingly, among 3 patients showed levels of NETest higher than 40%, two of them had R1 resection, and one had potentially nodal involvement. Among the remaining 15 patients, 12 exhibited a mean NETest level of 27 after resection (POD30), which is consistent with the presence of residual disease; even if the follow-up is not enough for identify recurrence it is possible to hypothesize that NETest score is able to identify patients at risk for recurrence [50]. In another study on gastric NENs, 5 patients underwent total gastrectomy and 8 subjects underwent partial gastrectomy. Despite all patients were disease free at endoscopical, radiological and functional imaging, NETest score was elevated in 6 patients, suggesting that these patients could have minimal residual disease. According to this hypothesis, the same study demonstrated that in case of microscopical residual disease evidenced at histological examinations (5 patients with positive polypectomy margin and 4 patients with random biopsies diagnostic for microscopic tumor) NETest was elevated (28 ± 9%), demonstrating the accuracy of this diagnostic instrument in detecting minimal residual disease before morpho-functional imaging [51].
In a recent study, Modlin et al. evaluated NETest in a cohort of 153 patients affected by surgically treated pulmonary and GEP neuroendocrine tumors. NETest was evaluated preoperatively, and on POD1 and on POD30. In the R0 cohort, POD30 NETest levels decreased but remained elevated (<20%) in 31 patients, and 25/31 patients with a POD30 NETest higher than 20% developed image-identifiable recurrence by the following 18 months. On the other side, all the patients with POD30 NETest values lower than 20% were free from recurrence during follow up. The authors demonstrated that a NETest value higher than 20% on POD30 predicted residual disease with 94% accuracy and 100% sensitivity [52].
Finally, the role of NETest in evaluating the efficacy of surgical excision has been confirmed also in pulmonary NEN. In the study by Filosso *et al.*, in a subgroup of 19 patients with pulmonary NEN (12 typical carcinoids, 4 atypical carcinoids, 3 large-cells neuroendocrine carcinoma) the NETest score significantly decreased 30 days after surgery (from 69 ± 28% to 29 ± 9%; mean reduction from baseline -59%). This reduction was present only in the cohort of NENs (differently from what observed in patients affected by other non-neuroendocrine pulmonary cancers, also enrolled in the study); 95% of surgically treated patients were R0 at histological examination but no data on longer follow-up or radiological examinations were provided [38].
The 6 prospective and 2 retrospective studies reporting data on NETest after surgical intervention are summarized in Table 3. In conclusion, NETest values between 20 and 40% can identify minimal residual disease in patients after apparently radical surgery. In this view, patients with negative NETest could need less close follow-up, reducing the number of total body imaging (PET or CT) with clear advantages from an economic point of view but also for the lower exposure to radiation [52], in a personalized medicine perspective [53].

Future: beyond NETest

In addition to the diagnostic and prognostic role of NETest, the evaluation of tumor transcription may provide additional information. In a study on 20 patients affected by SINETs, a specific subgroup of profibrotic circulating transcripts, the "fibrosome" of the NETest was able to predict mesenteric fibrosis in 100% of cases, even when conventional radiology was negative, providing important information to the surgeon [54]. In this sense NETest, as other liquid biopsy, may be considered a window on the tumor, providing different types of information.
Moreover, the research on NETest is continuing to develop. In 2020 Kidd *et al.* evaluated the expression of NET-omes and their combinations in a cohort of 88 patients affected by G1 and G2 GEP-NET, with the aim to identify pathologically relevant-omes for defining of disease status, and to investigate if these elements could provide

added prognostic information to the “classical” NETest score. Scores were assessed at baseline and after a median follow-up of 9 months. 4 NET -omes among those analyzed showed a prognostic value, defined as correlation between basal levels and outcome (PROGNOSOME: Metastasome, Epigenome, Fibrosome and NEDome). Then, the authors further investigated the prognostic role of NETest integrated with Prognosome levels. They found that the association at baseline between a low NETest score (<40) and PROGNOSOME levels below the upper limit of normal was an accurate prognostic factor for SD during follow-up (90%); on the contrary, high NETest score (>40) associated with prognosome levels above the upper limit of normal predicted PD within 3 months (100%). Integrating the 4 -omes with the NETest score (using 40% as a cut-off) generated an overall prognostic accuracy of 93%, significantly better than the prognostic value of either the NETest alone (70,5%) or the -omic analysis as a separate approach (69%) [55].

CONCLUSIONS

Prognostic assessment by NETest: ready for clinical application?

Prognostic markers in the field of NENs are lacking and necessary. NETest is the most characterized and validated application of the liquid biopsy to the field of NENs, reported as a key diagnostic advantage, and a promising tool for clinical practice [56]. Beyond its diagnostic value, is also a prognostic tool. In fact, a recent metanalysis considering data from 6 studies estimated the accuracy of NETest of 84.5%-85.5% in differentiating stable from progressive disease [20]. By this systematic review, we tried to answer specific questions which can be useful for clinical practice, as summarized in Figure 2. The two fields with more available data on the prognostic value of NETest are: the ability of NETest in differentiating SD from PD at baseline and the capacity to predict surgical treatment outcome. In particular, high levels of NETest (at least >40%) identify PD. Consequently, NETest could be useful in all situations in which is important to identify progression, as, for example, in case of new diagnosis of NEN, when it is not possible to establish disease course because previous imaging is clearly unavailable. High baseline NETest levels predict also a subsequent progression, even in case of SSA treatment. These patients should be probably evaluated frequently and treated in a more aggressive way, even if data seem not enough strength for applying this indication to clinical practice.

Finally, given the possibility to assist to a recurrence after an apparently radical surgical procedure, another application of NETest can be the identification of minimal residual disease. Probably, only negative value of NETest (<20%), defined as values found in healthy controls [31], correctly identified radical surgical treatment. In this sense, NETest can evaluate patients at high risk of recurrence who could benefit from a more frequent radiological examination or an adjuvant approach. In authors’ opinion, the introduction of NETest in clinical practice should become from this last application: to identify patients at high risk of recurrence despite the radical surgery, confirmed by no tumor infiltration in the resection margins at histological examination. In this setting, NETest seems not only informative but also able to reduce medical costs through the possibility to reduce the number of morphological and functional imaging during follow-up.

Statements

Statement of Ethics

An ethics statement is not applicable because this study is based exclusively on published literature.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions Statement

GP: writing of the original draft, data collection, data analysis and interpretation; VDV: writing of the original draft, data collection, data analysis and interpretation; TF: Data analysis and interpretation; FS: Data analysis and interpretation; RC: Data analysis and interpretation; CP: Data analysis and interpretation; MGT: Data analysis and interpretation; MV: critical revision of the article; AL: critical revision of the article; AMI: critical revision of the article; EG: supervision, critical revision of the article; AF: Conceptualization, critical revision of the article.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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Figure Legends

Fig. 1. Flow diagram of included and excluded studies. *respectively on Pubmed, Embase, Cochrane Library and Scopus. No time restriction has been applied in the search strategy; papers included in the systematic review have been published from 2015 to 2021.

Fig. 2. Possible clinical applications of NETest. Summary of questions and answers debated in the systematic review.

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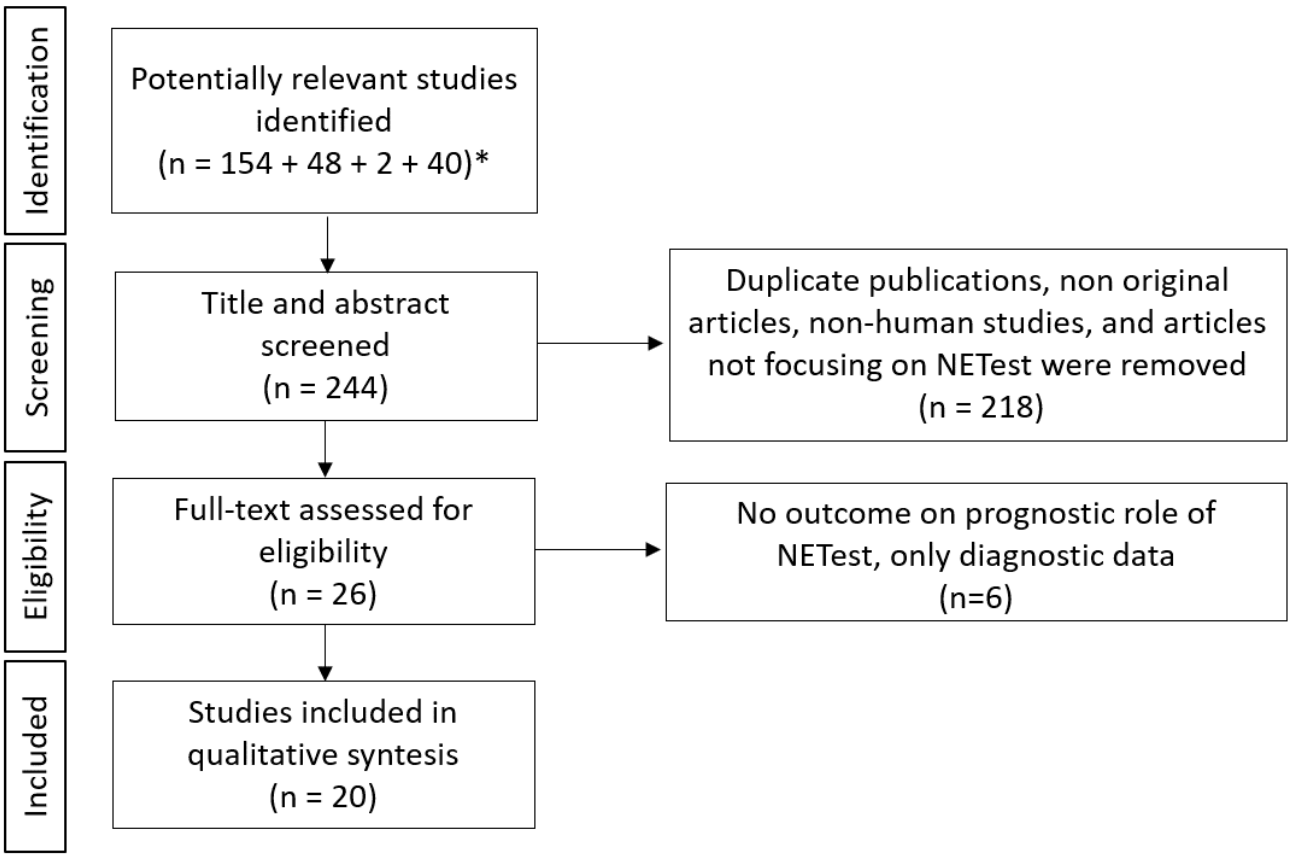
Identification
Potentially relevant studies identified (n = 154 + 48 + 2 + 40)*
Screening
Title and abstract screened (n = 244)
Duplicate publications, non original articles, non-human studies, and articles not focusing on NETest were removed (n = 218)
Eligibility
Full-text assessed for eligibility (n = 26)
No outcome on prognostic role of NETest, only diagnostic data (n=6)
Included
Studies included in qualitative syntesis (n = 20)

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NETest

1) Is NETest able to differentiate stable from progressive disease at the time of sampling?

Yes. A cut-off of 40-45% is able to differentiate SD from PD.
In case of higher cut-off (>80%) the percentage of PD clearly further increase

2) Is NETest able to predict tumor progression and tumor response to treatment?

Probably (few data available). NETest score >33-40% predicts tumor progression. NETest score decrease or stability predicts tumor response to treatment, while an increase predicts tumor progression under treatment (even before radiological findings)

3) Is NETest able to predict tumor recurrence after surgery?

Yes. Post-operative NETest score >33-40% is associated to tumor recurrence. Probably only negative values (<20%) correctly identifyed minimal residual disease

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Table 1. Articles evaluating the role of NETest in differentiating stable disease from progressive disease, assessed by morphological imaging, at the time of blood collection.

1: other sites include cancer of unknown primary origin (n = 7), ovary, vulva, vagina, and pituitary (n = 2). **2**: 55/119 patients with detectable disease underwent watchful waiting, while 64/119 were treated. **Abbreviations Table 1**: **GEP:** gastroenteropancreatic**; N/A:** not available**; CHT:** chemotherapy**; SSA:** somatostatin analogues**; SD:** stable disease; **PD:** progressive disease**; NF:** non functioning; **F:** functioning; **PRRT:** peptide receptor radionuclide therapy; **RT:** radiotherapy; **LCNEC**: large cell neuroendocrine carcinoma**; SCLC**: small cell lung carcinoma; **BP**: bronchopulmonary**; NEN**: neuroendocrine neoplasia; **TC:** typical carcinoid; **AC:** atypical carcinoid**; NET:** neuroendocrine tumor**; NEC:** neuroendocrine carcinoma**; LRT:** locoregional therapy; **PTS:** patients; **PPGLs:** pheochromocytomas/paragangliomas.

hor, ef]	Study design	Total number of patients	NENs localization (n)	Histology and grading	Mean age (range min-max)	Sex	Functionality status (NF/F)	Treatment before study entry	NETest values in SD *vs* PD (p value) Cut-off (sensitivity and specificity)	Quality assessment (MINORS)
	Prospective Cohort study	222	**TEST SET (63)** GEP (60)	G1 30; G2 18; G3 2; N/A 13.	56 (18–80)	M 33 F 30	N/A	Surgery; Interferon; CHT; SSA.	**SD:** 34.1±27% **PD**: 83.7±24.4% (p<0.0001) **Cut-off:** 45% (Sensitivity 91.1%, specificity 85.3%)	**10**
			Lung (3)							
			INDEPENDENT SET (159) GEP (141)	G1 87; G2 29; G3 5; N/A 38.	57.1 (27-83)	M 86 F 73	N/A			
			Lung (5)							
			N/A (12)							
	Prospective Cohort study	63	**Test set, basal (35)** GEP (35)	NET (grade NA)	58 (33–82)	M 12 F 23	N/A		**SD:** 32±19% **PD**: 82%±12% (p<0.0001) **Cut-off:** 80% (Sensitivity >80%, specificity >95%)	**9**
	Prospective Cohort study	34	Gut (25) Pancreas (9)	G1 17; G2 14; G3 1; N/A 2.	60.2 (43 – 83)	M 17 F 17	N/A	None 14; SSA 16; SSA+Everolimus 1; Streptozotocin/5-fu 3.	**SD:** 41.6±5.8% **PD**: 67.2%±7.1% (p<0.05) **(No cut-off proposed)**	**12**
	Retrospective and prospective Cohort study	226	Lung (set 1, diagnostic cohort: 131)	Typical carcinoids; atypical carcinoids; LCNEC; SCLC	BP carcinoids: 58.2 (21–79) Other lung NENs: 63 (47–73)	BP carcinoids: 45 M; 73 F Other lung NENs: 7 M; 6 F	N/A	N/A	**SD:** 36±19% **PD**: 73%±22% (p<0.001) **(No cut-off proposed)**	**12**
	Pilot cohort study	25	Lung (25)	TC 18; AC 7.	62 (46-77)	M 4 F 21	N/A	SSA 5; CHT 1.	**SD:** 32±7% **PD**: 85%±11% p<0.0001 **(No cut-off proposed)**	**7**
M,	Prospective Cohort study	32	PPGLs	Localized 18; multicentric 7; metastatic 4	34 (12-62)	M 17 F 15	NF 17 F 15	Surgery 25; embolization 5; PRRT 5; EBRT 3; brachytherapy 1.	**SD:** 41±5% **PD**: 86%±2% (p< 0.0001) **Cut-off:** 53% (Sensitivity 100%, specificity 85.7%)	**5**
	Prospective Cohort study	100	GEP (68)	G1 34; G2 13; G3 2.	61.5 (14–83)	M 34 F 66	N/A	Surgery: 69	**NETest < 40%:** SD in 54 pts (87% **NETest > 80%:** PD in 21 pts (81%)	**8**

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Table 2. Articles evaluating the role of NETest in predicting tumor disease progression and tumor response to treatment. Abbreviations: N/A: not available**; SSA:** somatostatin analogues**; PFS:** progression free survival; **PD:** progressive disease**; GEP:** gastroenteropancreatic**; PRRT:** peptide receptor radionuclide therapy; **CHT:** chemotherapy**; TACE:** transcatheter arterial chemoembolization**; SD:** stable disease; **PPGLs:** pheochromocytomas/paragangliomas; **NF:** non functioning; **F:** functioning; **EBRT:** external beam radiation therapy; **RT:** radiotherapy.

Study design	Total number of patients	NENs localization (n)	Histology and grading	Mean age (range min-max)	Sex	Functionality status (NF/F)	Treatment before study entry	Time of NETest	Role of NETest as predictor of progression and treatment response	Quality assessment MINORS
Prospective Cohort study	63	Prospective set (28) GEP (25) N/A (3)	G1 12; G2 16.	60 (36-81)	M 10 F 18	N/A	Surgery 25; SSA 28; PRRT 11; CHT 5; TACE 1.	**Basal** **T1:** every 4 weeks during SSA treatment	**Subsequent SD** → baseline NETest 41 ± 2% **Subsequent PD** →baseline NETest 57.5 ± 2% (if>80%: subsequent PD in 100% of patients) Mean NETest during SSA treatment>80% → non responders to SSA	**9**
Prospective Cohort study	54	GEP (35) Lung (13) N/A (6)	G1 6; G2 20; G3 3; N/A 6 TC 1; AC: 7; high grade 4; NA 1	66 (43–83)	M 37 F 17	NF 33 F 21	Surgery 32; SSA 44; CHT 21; Everolimus 5; Sunitinib 1; Interferon-alfa 1; PRRT 16; RT 6; TACE 4.	**Basal:** pre PRRT **T1:** +6 months follow up	**NETest (Basal-T1**): negative in 88% of responders; null or positive in 90% of non-responders.	**11**
Prospective Cohort study	34	Gut (25) Pancreas (9)	G1 17; G2 14; G3 1; N/A 2.	60.2 (43 – 83)	M 17 F 17	N/A	None 14; SSA 16; SSA+Everolimus 1; Streptozotocin/5-fu 3.	**Basal**	**Basal NETest <40%** → PFS 2.78 years **Basal NETest >80%** → PFS 0.68 years Basal NETest > 40% predicts subsequent PD in 7/7 pts (100%)	**12**
Prospective Cohort study	32	PPGLs	Localized 18; multicentric 7; metastatic 4	34 (12-62)	M 17 F 15	NF 17 F 15	Surgery 25; embolization 5; PRRT 5; EBRT 3; brachytherapy 1.	**Basal** **T1**: +2/12 months follow up (in 9 pts)	**Basal NETest > 53%** was associated to disease progression **NETest score variations** during follow-up were associated to disease status.	**5**
Prospective Cohort study	100	Watch and wait cohort Treatment cohort	N/A					**Basal**	**Basal NETest <40%** → PFS 12 months **Basal NETest >80%** → PFS 3 months <40% → responders to SSA >80% → non responders to SSA	**8**
Prospective Cohort study	152	GEP	G1 105; G2 44; G3 2; N/A 1.	53 (25–81)	M 82 F 70	N/A	N/A	**Basal:** pre-therapy	**All patients:** **Basal NETest <33%**→ PFS 55 months **Basal NETst 34–79%**→ PFS 18 months **Basal NETest > 80%**→ PFS 11 months **Watchful waiting group (88)** **Basal NETest<33%** → PFS 54 months	**13**

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Table 3. Articles evaluating the role of NETest in predicting tumor recurrence after surgery. Abbreviations: **LCNEC**: large cell neuroendocrine carcinoma**; N/A:** not available**; GEP:** gastroenteropancreatic**; NF:** non functioning; **F:** functioning; **POD:** post-operative day; SSA: somatostatin analogues; CHT: chemotherapy; PRRT: Peptide Receptor Radionuclide Therapy;

Study design	Total number of patients	NENs localization (n)	Histology and grading	Mean age (range min-max)	Sex	Functionality status (NF/F)	Type of surgery/resection	Time of NETest	Role of NETest as predictor of recurrence after surgery (cut-off for minimal residual disease)	Quality assessment (MINORS)
Retrospective and prospective Cohort study	226	Lung (set 2, surgical cohort: 19)	Typical carcinoids; atypical carcinoids; LCNEC.	62 (34–82)	M 8 F 11	N/A	Lung surgery 19	**Basal**: before surgery **T1**: +30 days surgery	**Mean ΔNETest: -59% (95% R0 at histological examination)**	**12**
Prospective Cohort study	35	GEP (35)	G1 27; G2 7; G3 1.	55.7 (33-80)	M 14 F 21	N/A	Surgery R0 15; surgery R1 12; ablative resection 8.	**Basal:** pre-surgery **T1:** +1month surgery	**Cut off for minimal residual disease 14%**	**9**
Prospective Cohort study	13	Small intestine (13)	G1 8; G2 5.	64 (48-79)	M 10 F 3	N/A	Surgery 13	**Basal:** pre-surgery **T1:** follow up post-surgery (median 22 months)	**Cut off for minimal residual disease 40%**	**8**
Prospective Cohort study	35	**R0 with no recurrence** Pancreas (11)	G1 8; G2 3.	63 (59-65)	M 4 F 7	NF 11 F 0	Surgery 35	After surgery	**Cut off for minimal residual disease 40%**	**10**
		R0 with recurrence Pancreas (12)	G1 5; G2 7.	62.5 (61-64)	M 6 F 6	NF 11 F 1				
		R1 with no recurrence Pancreas (12)	G1 12	57 (50-59.5)	M 6 F 6	NF 7 F 5				
Prospective Cohort study	30	Pancreas (30)	G1 12; G2 17; G3 1	54 (40.6-67.4)	M 11 F 19	NF 22 F 8	Surgery 30	**Basal:** pre-surgery **T1:** POD 30	**Cut off for minimal residual disease 27%**	**9**
Retrospective Cohort study	46	Stomach	G1 32; G2 10; G3 1; NEC 3	55 (28-84)	M 13 F 33	NF 46 F 0	Polypectomy 24; partial gastrectomy 8; total gastrectomy 5; none 1.	**T1**: post-surgery	**Cut off for minimal residual disease 20%** **NETest in image negative R1 patients**: 28 ± 9% (100% > 20%) **NETest in image negative R0 patients**: 16 ± 11% (p=0.02)	**12**
Prospective Cohort study	153	Pancreas (57) Small intestine (62) Lung (27) Duodenum (4) Stomach (3)	G1:29; G2: 27; NET G3:1 G1: 40; G2: 22 TC: 17; AC: 10 G1: 3; G2: 1 G1: 2; NET G3: 1	58 (19-84)	M 74 F 79	NA	Surgery 153	**T0:** pre-surgery **T1:** POD 1 **T2:** POD 30	**Cut off for minimal residual disease 20%** (Sensibility: 100%; specificity: 92%, AUC: 0.97±0.02, p>0.0001) R0 cohort (102): 25/31 with POD30 NETest > 20% developed recurrence	**14**

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