5 Circulating Biochemical Markers of Gastro-Entero-Pancreatic (GEP) Neuroendocrine Neoplasms (NENs)

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5.1 Introduction

Neuroendocrine neoplasms (NENs) are a heterogeneous group of rare malignancies which represent a true challenge for clinicians at all stages of the disease, from diagnosis to treatment. The term "neuroendocrine" adequately describes the cell features, characterized by the presence of dense-core granules, similar to those found in serotonergic neurons, which is the reason for the "neuro" term, whereas "endocrine" refers to the secretive properties of these tumors. NENs arise from neuroendocrine cells which are derived from the diffuse endocrine system and represent approximately 2% of all malignant tumors of the gastro-entero-pancreatic (GEP) system [1]. Their incidence and prevalence have been increasing over the past years partly due to increased awareness and improvements in instrumental diagnostic techniques. NENs are usually divided into functioning and nonfunctioning forms. Functioning tumors usually synthesize, store, and secrete peptides and neuroamines that can cause distinct clinical syndromes, while nonfunctioning forms are clinically silent, being lately diagnosed once metastatic with mass effects [2].

Management of NEN represents a clinical challenge because of its late presentation, scarcity of standardized treatment options, and limitations in present imaging modalities and biomarkers to guide management. Biochemical markers are evaluated in the blood, urine, or other body fluids and are usually elevated in the presence of a tumor [3]. Of note, the beginning of the diagnostic process of NENs is often based on the measurement of circulating markers, before planning expensive and invasive diagnostic tests [4, 5]; however up to 60–80% of NENs are metastatic at diagnosis, which highlights the frequent failure to identify symptoms or to establish a biochemical diagnosis [2]. Furthermore, the majority of available markers, which can be divided into general and specific biomarkers, lack sensitivity and/or specificity and are often not helpful in the diagnostic process. A multinational consensus meeting of multidisciplinary experts in NENs assessed the use of current biomarkers and defined the perquisites for novel biomarkers via the Delphi method. Consensus (at >75%) was achieved for 88 (82%) of 107 assessment questions. The panel concluded that circulating

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multianalyte biomarkers provide the highest sensitivity and specificity necessary for minimum disease detection and that this type of biomarker had sufficient information to predict treatment effectiveness and prognosis. The panel also concluded that no monoanalyte biomarker of NENs has yet fulfilled these criteria and there is insufficient information to support the clinical use of miRNA or circulating tumor cells as useful prognostic markers for this disease.

The identification of biomarkers of both diagnostic and prognostic value for NENs is urgently needed to improve patient management and tailor the therapeutic approach for each patient [6].

5.2 Specific Biomarkers

Specific biomarkers are secreted by specialized neuroendocrine cells by functioning NEN and are responsible for specific GEP-NEN associated clinical syndrome. Specific markers include 5-hydroxyindole acetic acid (5-HIAA), insulin, gastrin, vasoactive intestinal peptide (VIP), glucagon, growth hormone-releasing hormone (GHRH), calcitonin, adrenocorticotrophic hormone (ACTH), and corticotropin-releasing hormone (CRH). The main features of the established functioning neuroendocrine syndromes have been reported in Table 5.1. Also, several other biologically active substances may be released from NENs such as bradykinin, substance P, neurotensin, human chorionic gonadotropin, neuropeptide K, and neuropeptide L.

5.2.1 5-Hydroxyindole Acetic Acid (5-HIAA)

5-hydroxyindole acetic acid (5-HIAA) is the urinary metabolite of serotonin or 5-hydroxytryptamine (5-HT) a peptide mainly synthesized and stored in the enterochromaffin cells of the gastrointestinal (GI) tract (80% of total body serotonin) [7], as well as in the serotoninergic neurons of the central nervous system [8] and the platelets. Serotonin is involved in different biological functions including vasoconstriction, neurotransmission, regulation of sleep, appetite, and gastrointestinal motility [9].

Hypersecretion of 5-HT and other biologically active amines (such as tachykinins, prostaglandins, and bradykinins) is usually observed in the presence of a metastatic small intestine NEN and results in a typical carcinoid syndrome. Elevated 5-HIAA levels in the urine are highly suggestive of an ileal NEN (approximately 75% of midgut NENs are associated with a positive urinary 5-HIAA test), although some NENs found in the lung and pancreas also secrete serotonin [9, 10]. The typical presentation is characterized by flushing, diarrhea, and abdominal pain. Less frequent symptoms are bronchospasm, headache, hypotension, lacrimation, profuse sweating, and cutaneous manifestations pellagra-like due to lack of niacin (Fig. 5.1) [5, 11, 12]. In about 10–20% of patients, carcinoid syndrome may lead to carcinoid heart disease in which cardiac fibrosis and thickening of the heart valves result in right heart failure [13]. The presence of heart disease confers a significantly worse prognosis, thus initial screening and multidisciplinary assessment are essential for both controlling carcinoid and cardiovascular symptoms and determining a strategy for medical and surgical management [14].

While 5-HT measurement is not recommended due to fluctuations in secretion as well as wide interindividual variations, the urinary 24-h measurement of 5-HIAA is a useful specific marker for 5-HT secreting NENs. Samples should be collected for 24 h using plastic jars shielded from light and prefilled with an acidic additive to keep pH below 3 (to ensure sterility and stability) [15]. Reliable methods for 5-HIAA determination are high-performance liquid chromatography (HPLC), automated assays, and mass spectrometry [16]. 5-HIAA presents a high intraindividual variability, thus a mean of two consecutive 24-h collections should be taken as reference [9]. The overall sensitivity and specificity of urinary 5-HIAA in the presence of the carcinoid syndrome are up to 90% [17]. However, as for most biomarkers, 5-HIAA presents false-positive and false-negative results. 5-HIAA levels

Table 5.1 Main epidemiological and biochemical features of the established functioning neuroendocrine syndromes in adults

Tumor	Markers	Tumor location	Incidence (cases/1000000/year)	Clinical syndrome
Carcinoid	Urinary 5-HIAA, serum 5-HIAA (less reliable)	Small intestine (60%) Colon-rectum (10%) Lung (30%) Pancreas (<1%)	3–11	Carcinoid syndrome
Insulinoma	Insulin (72 h fasting), C-peptide, proinsulin glucagon stimulation test	Pancreas (>99%)	1–3	Hypoglycemic symptoms
Gastrinoma	Gastrin, secretin stimulation test, calcium stimulation test, glucagon stimulation test	Duodenum (65%) Pancreas (30%) Other sites (5%)	0.5–2	Recurrent peptic ulcers Gastroesophageal reflux Diarrhea
VIPoma	VIP	Pancreas (90%) Other sites (10%)	0.05–2.0	Watery diarrhea Hypokalemia Achlorhydria
Glucagonoma	Glucagon	Pancreas (100%)	0.01–0.1	Necrolytic migratory erythema Diabetes mellitus Muscle wasting Weight loss
Somatostatinoma	Somatostatin	Pancreas (55%) Duodenum/small intestine (44%)	0.04	Diabetes mellitus Diarrhea cholelithiasis Weight loss Hypochlorhydria
ACTHoma	ACTH, cortisol	Lung (35%) Pancreas (25%) Thyroid (20%) Pheochromocytoma (10%) Other sites (10%)	Rare	Cushing's syndrome
CRHoma	CRH ACTH Cortisol	Thyroid (33%) Pheochromocytoma (19%) Lung (10%) Small intestine (5%)	Rare	Cushing's syndrome
GRHoma	GRH	Lung (54%) Pancreas (30%) Small intestine (7%) Other sites (13%)	Rare	Acromegaly
Calcitoninoma	Calcitonin	Pancreas Lung Pheochromocytomas other sites	Rare	Diarrhea

5-HIAA 5-Hydroxyindole acetic acid, *GRH* growth hormone-releasing hormone, *ACTH* adrenocorticotropic hormone, *CRH* corticotropin-releasing hormone, *VIP* vasoactive intestinal peptide

depend on tumor burden and can be normal in nonmetastatic patients [16]. NEN localization also influences urinary 5-HIAA levels; the sensitivity is lower in patients with fore- and hindgut NENs due to less serotonin production from these tumors than midgut forms. Moreover, renal failure and/or hemodialysis could result in falsely low 5-HIAA levels [10]. Somatostatin analogs are known to decrease levels of 5-HIAA, similarly other medications such as levodopa, methyldopa,

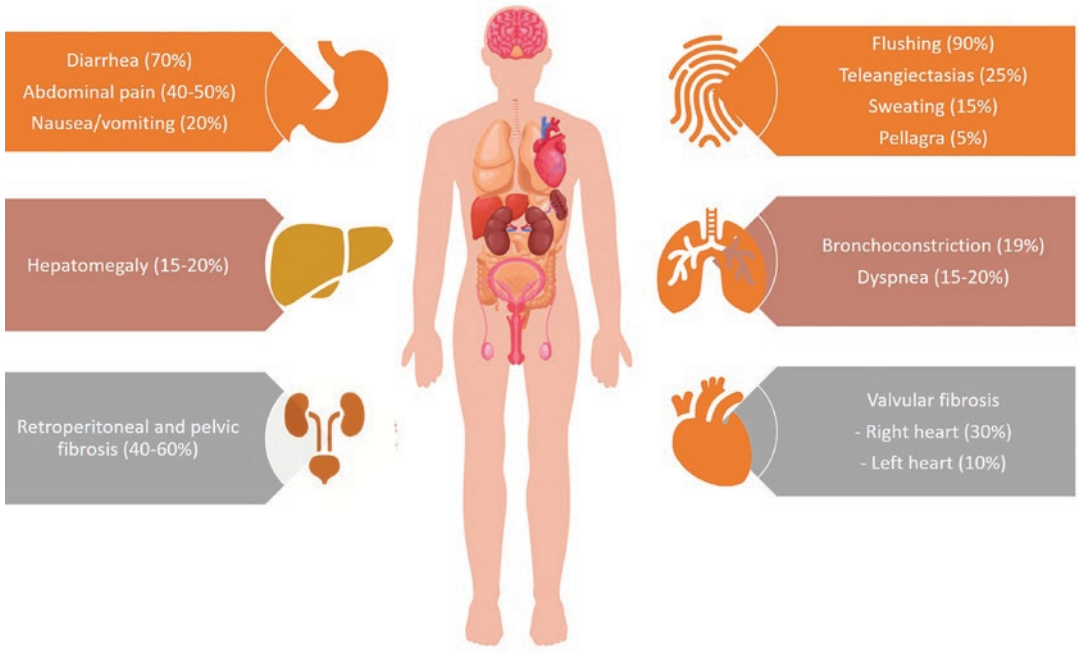

Fig. 5.1 Main manifestations of carcinoid syndrome and their relative frequencies

acetylsalicylic acid, adrenocorticotrophic hormone (ACTH), and phenothiazines may give false-negative results [18].

False-positive results can be observed in the presence of malabsorptive condition (i.e., celiac disease, tropical sprue, Whipple disease, intestinal stasis, and cystic fibrosis) or due to consumption of tryptophan/serotonin-rich food collection (i.e., tomatoes, plums, pineapples, bananas, eggplants, avocados, and walnuts) [18]. A three-day diet free of food rich in tryptophan/serotonin is advised to avoid false-positive results [9, 19].

A prognostic value of 5-HIAA in patients with carcinoid syndrome has been proposed. Different studies reported high 5-HIAA levels to be associated with a worse prognosis [20, 21], as well as a shorter 5-HIAA doubling time [20]. Moreover, a strong correlation between 5-HIAA circulating levels and carcinoid heart disease onset and progression has been observed.

5.2.2 Insulin

Insulin is a polypeptide composed of 51 amino acids produced in the pancreatic islets of Langerhans from β cells. The active form of insulin is synthesized from the proinsulin precursor molecule and consists of two peptide chains, the A-chain and B-chain [22]. Insulin plays a key role in energy balance and glucose metabolism mainly reducing blood glucose levels, by increasing glycogen synthesis and promoting the storage of glucose in the liver (and muscle) cells.

Thus, an inappropriate secretion of insulin, observed in presence of insulin-producing tumors or insulinomas, results in hypoglycemia. The low level of blood glucose accounts for the typical clinical features including both adrenergic activation (palpations, sweating, pallor, anxiety) and neuroglycopenic symptoms (personality changes and loss of consciousness) [9]. Insulinomas arise almost exclusively from the

pancreas and represent the most common pancreatic functioning NENs [9]. Insulinomas are usually present as small, hyper-vascularized neoplasms and may occur sporadically in up to 90% of cases or as part of multiple endocrine neoplasia type 1 (MEN1) syndrome in about 10% of the cases [23].

Insulinoma should be suspected in the presence of the Whipple's triad: symptomatic episodes of hypoglycemia, demonstration of serum glucose level <2.5 mmol/l (45 mg/dl), and relief of symptoms following glucose administration [24]. The biochemical diagnosis requires the presence of hypoglycemia <2.5 mmol/l (45 mg/dl) along with evidence of inappropriately increased insulin levels (>6 U/L) and C-peptide and proinsulin, which can be demonstrated in blood samples. Of note, insulin concentrations may be within the reference range; however, insulin is inappropriately high for the blood glucose level. In the presence of an episode of spontaneous severe hypoglycemia with hyperinsulinism, the simultaneous measurement of serum C-peptide and beta-hydroxybutyrate is appropriate [17]. The gold standard for the diagnosis is a 72-h fasting test and it attests autonomous insulin secretion and the failure of appropriate insulin suppression in the presence of hypoglycemia. The test requires the hospitalization and placement of an intravenous line as the patient undergoes a blood sampling for serum glucose and insulin every 6 h, or whenever symptoms of hypoglycemia occur. The test is suspended when plasma glucose falls below the threshold of 55 mg/dL, and the patient develops symptoms of hypoglycemia. Hypoglycemia develops within 12 h in 30% of patients, in 90% within 48 h, and approaches 100% within 72 h [9]. If the 72-h fasting test is not conclusive despite a strong clinical suspicion, a glucagon stimulation test can be performed immediately after the 72-fasting test: glucagon 1 mg is administered intramuscularly with a consequent increase in serum glucose levels that demonstrates adequate glycogen stores and is usually observed in patients with insulinoma.

The differential diagnosis of insulinoma includes abuse of insulin, sulphonylurea, or related insulin secretagogues and the use of hypoglycemic medications in the setting of renal impairment [9, 23].

5.2.3 Gastrin

Gastrin is an aminoacidic peptide physiologically involved in the stimulation of gastric acid (HCl) secretion and gastrointestinal motility. Gastrin is synthesized by G cells in the gastric antrum, duodenum, and the pancreas as a large precursor, progastrin. After cleavage and processing, progastrin is metabolized in several biologically active peptides including gastrin 34, gastrin 17, and C-terminally extended gastrins [25]. The release of gastrin is stimulated by food and inhibited by a low gastric pH. Gastrin binds to the cholecystokinin-2 receptor regulating the meal-stimulated gastric acid secretion. Besides, it plays important roles in epithelial cell proliferation in the gastrointestinal tract [25].

Gastrin-producing tumors, named gastrinomas, are the second most common functioning NENs. They usually arise in the duodenum (50–70% of cases) or the pancreas (20–40%) in a small portion called the gastrinoma triangle [26]. Gastrinomas can be sporadic or occur as part of MEN1 syndrome (approximately 25–35%). Thus, in case of gastrinoma diagnosis, screening for MEN1 is, therefore, advisable [16].

Hypersecretion of gastrin from gastrinoma leads to Zollinger-Ellison syndrome (ZES) characterized by increased gastric acid production from fundic parietal cells [27]. The excess in gastric acid secretion causes severe recurrent peptic ulcer disease and inactivates pancreatic digestive enzymes with consequent fat malabsorption and diarrhea. The inhibition of absorption of sodium and water by the small intestine results in a secretory diarrhea [28]. Malabsorption

and weight loss may occur in patients with long-standing untreated disease [29]. The diagnosis of gastrinoma is usually delayed of an average of 8 years from the start of symptoms to diagnosis, this is mostly due to the widespread use of proton pump inhibitors (PPIs) which can conceal ZES symptoms [30, 31].

Refractory gastric hyperacidemia, recurrence of ulcers despite maximal medical therapy, and presence of large, multiple ulcers should arise a high level of suspicion. Measurement of fasting serum gastrin is suggested to diagnose ZES: high gastrin levels (often ten times the upper normal value) and low gastric pH are required to perform diagnosis [8, 32]. However, 50–60% of patients with ZES have serum gastrin concentrations less than ten times the upper normal value (generally between 150 and 1000 pg/mL), so fasting gastrin alone is not adequate for a conclusive diagnosis of ZES. Moreover, hypergastrinemia may be observed in other conditions than ZES such as hypochlorhydric conditions (PPIs use, chronic atrophic autoimmune gastritis), antral G cell hyperplasia, gastrojejunostomy, hypercalcemia, and chronic renal impairment (Table 5.2). PPIs should be discontinued 2 weeks before serum gastrin evaluation, a switch to high doses of H2 blockers is recommended in order to prevent peptic complications [16, 33, 34].

Provocative tests can be used for gastrinoma diagnoses when serum fasting gastrin is mildly increased, or in patients undergoing PPIs treatment [9]. The secretin stimulation test is the most used provocative test for the diagnosis of gastrinomas having high sensitivity and specificity (94% and 100%, respectively) and can differentiate patients with gastrinomas from those with hypergastrinemia from different causes. The test consists of the administration of secretin (2 U/kg body weight) by intravenous bolus; serum gastrin is measured at baseline (15 and 1 min before the test) and then 2, 5, 10, 15, 20, and 30 min after secretin administration. An increase of ≥120 pg/mL at any time during the test confirms the diagnosis [16, 17] (Fig. 5.2). Additional stimulation tests can be considered in the case of an inconclusive secretin test. The calcium stimulation test is the most used in the presence of high clinical suspicion for ZES with a negative secretin test [35]. Serum gastrin is assessed every 30 min after the administration of calcium gluconate (5 mg/kg) over 3 h. An increase in serum gastrin >20% from baseline, usually with gastrin above 300 pg/mL, is conclusive for diagnosis. Additionally, the glucagon test is used for the diagnosis of gastrinomas. Glucagon is infused at 20 μg/kg/h for 30 min; an increase over the baseline within 10 min in presence of circulating gastrin over 200 pg/mL is suggestive for gastrinoma [36]. The glucagon test can also be used postoperatively, as a measure of surgical efficacy: a negative response representing a sign of adequate tumor removal and being associated with a decreased chance of recurrence [37]. Finally, the basal acid output

Table 5.2 Conditions associated with hypergastrinemia in relation to gastric acid secretion

Decreased acid secretion (pH > 5)	Normal or increased acid secretion (pH < 5)
Chronic autoimmune atrophic gastritis	Gastrin secreting tumor
Proton pump inhibitors/H-2 blockers intake	Antral G cell hyperplasia
H. pylori infection	Duodenal ulcer
Vagotomy	Retained antrum syndrome
Gastric cancer without the involvement of the gastric antrum	Pyloric stenosis
	Hypercalcemia
	Massive bowel resection
	Chronic renal impairment

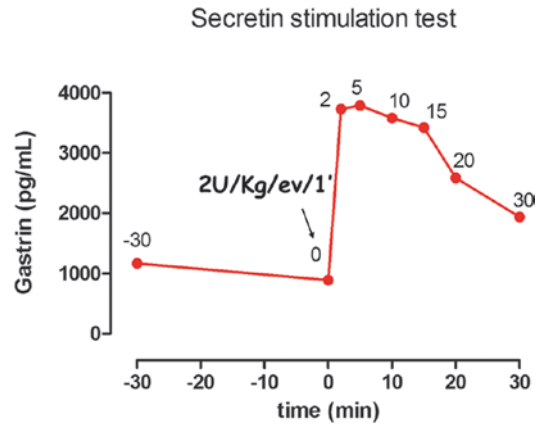

Fig. 5.2 Positive secretin stimulation test in a patient affected by gastrin secreting tumors. Plasma gastrin levels were measured at −30 and 0 time, and 2, 5, 10, 15, 20, and 30 min after intravenous secretin infusion

(BAO) can support the diagnosis of ZES: a BAO >15 mmol/h is suggestive for this diagnosis [9].

5.2.4 Vasoactive Intestinal Peptide (VIP)

Vasoactive intestinal peptide (VIP) is a neurotransmitter, belonging to the secretin-glucagon family, composed of 28 amino acids. VIP physiologically acts as a neuromodulator and not as a hormone. VIP is released from neurons and peripheral ganglia in several tissues throughout the GI tract, in the urogenital system, respiratory tract, blood vessel, and in the central nervous system in the suprachiasmatic nuclei of the hypothalamus. On the digestive system, VIP has several effects: vasodilatation, smooth muscle regulation, stimulation of water and electrolyte secretion from the GI tract, inhibition of gastric acid secretion, and increase of blood flow in the GI tract. These effects work together to increase GI motility. Moreover, VIP promotes insulin and glucagon secretion [16].

VIP secreting tumors, namely VIPomas, are rare tumors occurring both in children and adults, with an incidence ranging from 0.05% to 2.0% [38]. In adults, they are mostly located in the pancreatic tail [39], while a small proportion of VIP secreting tumors has been reported in association to colorectal cancer, lung cancer, pheochromocytoma, neurofibroma, and ganglioneuroblastoma. The majority of VIPomas present as isolated tumors, but in about 5% of patients, they are part of the MEN1 syndrome [4]. More than 50% of VIPomas have metastasized by the time of diagnosis. In children, VIPomas are typically diagnosed at 2–4 years and typically occur in ganglioneuroma and neuroblastoma [40].

Due to VIP effects as a potent stimulator of intestinal secretion and inhibitor of gastric acid secretion, VIPoma is characterized by watery diarrhea, hypokalemia, and achlorhydria (hence WDHA syndrome or pancreatic cholera syndrome, also called Verner Morrison syndrome). In the WDHA syndrome, the watery diarrhea is chronic with a fasting stool volume from 750 to 1000 mL/day, resulting in dehydration, hypokalemia, achlorhydria, acidosis, hyperglycemia, and vasodilation. Watery diarrhea may be intermittent at the onset, but it can rapidly escalate and reach a volume of 15–20 L per day, causing profound fluid and electrolyte imbalance. Hypokalemic acidosis is due to bicarbonate and potassium loss across the bowel mucosa; it may provoke asthenia and tetanic contraction. Gastric achlorhydria occurs in 50% of patients only, while hypochlorhydria is usually present. Abdominal pain and weight loss are also common features. Vasodilation causing flushing and hypotension mimics the classical midgut carcinoid syndrome. Finally, hypercalcemia can be observed due to VIP direct action on bone metabolism [23].

In physiological conditions, VIP circulates in low quantities, so even increases of 20–50% can be significant, therefore it has a high specificity; however, data on its sensitivity are lacking.

5.2.5 Glucagon

Glucagon is a 29-amino acid peptide hormone secreted by pancreatic α cells and from the L cells in the intestinal mucosa. In the pancreas, proglucagon is processed to produce glucagon, glicentin-related peptide, intervening peptide, and the major glucagon fragment. Intestinal proglucagon undergoes alternative posttranslational processing that generates glicentin, glucagon-like peptide 1 (GLP1), and glucagon-like peptide 2 (GLP2) [4]. Glucagon's main action is to raise blood glucose levels, stimulating glycogenolysis and gluconeogenesis, with an opposite action compared to insulin. Glucagon is released in response to hypoglycemia, amino acid ingestions, increased catecholamines, and ghrelin. On the other hand, glucagon is inhibited by hyperglycemia, insulin, somatostatin, and GLP-1 [41, 42].

Glucagon secreting tumors, named glucagonomas, are rare tumors with an annual incidence ranging from 0.01 to 0.1 per 100,000 [43]. They typically arise from the tail or the body of the pancreas due to the high prevalence of alpha

cells in this area. More than 50% are metastatic at the time of diagnosis.

Excessive secretion of glucagon from the tumor causes a clinical syndrome called "4D syndrome," consisting of dermatosis (necrolytic migratory erythema), diabetes, deep vein thrombosis, and depression. Weight loss, diarrhea, and mucosal abnormalities (i.e., stomatitis, cheilitis, and glossitis) may also be observed [44–46]. Necrolytic migratory erythema, which is present in up to 90% of the patients, usually appears as an itchy rash on the perineum, thighs, and distal extremities prone to secondary infections. The pathophysiology of this dermatological manifestation has not been clarified, but it is thought to be secondary to a combination of poor nutrition, low zinc, and amino acid levels.

Elevated plasma glucagon levels, above 500 pg/ml (normal value <150 pg/mL), are usually observed only in the presence of glucagonomas [47]. Also, glicentin could be measured resulting markedly increased.

Mild elevation in glucagon levels can be observed in different conditions, such as cirrhosis, untreated diabetes mellitus, prolonged fasting, sepsis, burns, and Cushing's syndrome [17].

5.2.6 Somatostatin

Somatostatin is a peptide hormone secreted from the delta cells of the pancreas, the gastric antral D cells, and the APUD (Amine Precursor Uptake and Decarboxylation) cells [48]. Somatostatin acts on the anterior pituitary inhibiting the release of growth hormone (GH) and thyroid-stimulating hormone, adrenocorticotropic hormone (ACTH), and prolactin [49]. In the neuro GI system, somatostatin suppresses the secretion of several gastrointestinal and pancreatic hormones such as pancreatic polypeptide (PP), glucagon, cholecystokinin, gastrin, secretin, cholecystokinin, VIP, gastric inhibitory polypeptide, motilin, and neurotensin [49]. In addition, somatostatin has a direct inhibitory effect on gastric acid secretion and reduces smooth muscle contractions and bowel motility [4].

Somatostatinoma are rare neoplasms with an incidence of 1 in 40 million individuals [50]. They are localized in the pancreas in up to 70% of the cases, while other common sites include duodenum (19%), ampulla of Vater (3%), and small bowel (3%) [49, 51]. Reports exist of rare instances of extra-GI primaries [52]. Somatostatinoma can be sporadic or may occur in association with familial syndromes such as MEN1 (40 to 50% of cases), neurofibromatosis type 1, and Von Hippel-Lindau syndrome. The most common manifestations include cholelithiasis, which is present in almost 70% of the cases, and diabetes mellitus in 60% of the cases [51]. Rarely, somatostatinomas manifest as a triad of diabetes mellitus, cholelithiasis, and steatorrhea referred to as inhibitory syndrome due to the suppression of insulin, cholecystokinin, and pancreatic exocrine enzymes, respectively. Moreover, hypochlorhydria can be observed due to the inhibition of gastrin secretion [48, 49]. However, in most cases, somatostatinoma is detected in an advanced stage in presence of mass effect or in presence of metastases with clinical manifestations.

Somatostatinomas usually present elevated fasting serum somatostatin levels (greater than 14 mmol/l) [51]. However, serum somatostatin levels have been reported to be increased in other endocrine neoplasms such as medullary thyroid cancer, lung cancer, pheochromocytoma, and paraganglioma [48, 49].

5.2.7 Other Circulating Markers

Several peptide hormones have been reported to be secreted from NENs arising in different sites. Hereby we present the main circulating markers which have been recognized to cause a clinical syndrome. Besides, other peptides have been rarely reported to be secreted in NEN, even if often localized in extra GI and pancreatic site.

5.2.7.1 Adrenocorticotropic Hormone (ACTH)

Adrenocorticotropic hormone (ACTH) is a 39-amino acid hormone secreted from the

anterior pituitary gland. ACTH is part of the hypothalamic-pituitary-adrenal axis. It is synthesized in response to the hormone corticotropin-releasing hormone (CRH) released from the hypothalamus and acts on the adrenal gland increasing the production and release of cortisol.

For these reasons, an excess of ACTH leads to an increased secretion of cortisol outlining a Cushing syndrome. Typical features include muscle weakness, increased body weight, hypertension, hyperglycemia, hypokalemia, infections, bruising, osteoporosis, and psychiatric disorders [53].

ACTH ectopic secretion accounts for 10% to 20% of all cases of Cushing syndrome [53]. The source of ectopic ACTH syndrome is usually a small cell lung cancer, bronchial carcinoid, medullary thyroid cancer, and pheochromocytoma [54, 55].

5.2.7.2 Corticotropin-Releasing Hormone (CRH)

Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide derived from a 191-amino acid precursor. CRH acts as hormone and neurotransmitter on the posterior pituitary stimulating ACTH synthesis in stress response. CRH is produced by parvocellular neuroendocrine cells (contained within the paraventricular nucleus of the hypothalamus). CRH secreting tumors are rare, and they may occur in patients with medullary thyroid cancer (about 33%) and pheochromocytoma (19%), small-cell lung carcinoma (about 10%), and small intestine NEN (5%) [56, 57].

CRH secretion from tumors results in increased ACTH levels. Thus, the main clinical features are those of Cushing's syndrome as reported above. Levels of cortisol are elevated (>900 nmol/l) as ACTH, dehydroepiandrosterone sulfate (DHEA-S). Overnight administration of dexamethasone does not suppress cortisol secretion [56].

5.2.7.3 Growth Hormone-Releasing Hormone (GHRH)

Growth hormone-releasing hormone (GHRH) is a 44-amino acid hormone released from neurosecretory nerve terminals of the arcuate neurons in the hypothalamus and acts on the anterior pituitary, where it stimulates the secretion of growth hormone (GH).

An increase in GHRH levels results in GH hypersecretion and acromegaly. Several hypothalamic tumors, such as hamartomas, gliomas, and gangliocytomas, may produce GHRH. Peripheral GHRH levels are usually not elevated in patients with hypothalamic GHRH-secreting tumors, as GHRH secretion into the hypophyseal portal system does not appreciably enter the systemic circulation. Excessive ectopic peripheral production of GHRH has been reported in several tumors, including pulmonary NENs and small-cell lung cancers (54%), pancreatic NENs (30%), small-intestine NENs (7%), adrenal adenomas, and pheochromocytomas. In these cases, peripheral GHRH levels are usually elevated. GHRH plasma levels evaluation provides a precise and cost-effective test for the diagnosis of ectopic acromegaly. Thus, elevated circulating GHRH levels in presence of a non-enlarged pituitary gland should drive the suspect of extra-pituitary production of GHRH [58].

5.2.7.4 Calcitonin

Calcitonin is a 32-amino acid peptide released from non-follicular C-cells of the thyroid. It is produced as a 136-amino acid precursor (procalcitonin) and processed in secretory granules to the active form. The synthesis and release of calcitonin are closely related to calcium serum levels.

Inappropriate secretion of calcitonin results in hypercalcemia. Calcitonin is raised in medullary thyroid cancer, where concentration may be thousand-fold the reference range. Medullary thyroid cancers frequently arise as part of MEN type 2 (MEN2) syndrome. Also, calcitonin has been reported to be raised in other solid neoplasms including pancreatic and pulmonary NENs, pheochromocytomas, neuromas, breast, prostate, and colorectal carcinomas [59, 60]. Usually, ectopic-produced calcitonin is a large molecule without biochemical activity [4].

5.3 Nonspecific Biomarkers

Several families of secretory proteins can be found in high concentrations in neuroendocrine cells and, in particular, in NENs, and these include the granins, neuron-specific enolase (NSE), and pancreatic polypeptide (PP). The chromogranin family consists of at least three different water-soluble acidic glycoproteins [chromogranin A (CgA), chromogranin B (CgB), and secretogranin II, sometimes called chromogranin C]. Both CgA and NSE show increased concentration levels in many NEN patients. CgA is the most commonly used biomarker for NEN disease, although its utility is controversial [61]. However, CgA is the only general biomarker that has been extensively investigated [61–63].

5.3.1 Chromogranin A (CgA)

Chromogranin A (CgA), which is an acidic glycoprotein of 439 amino acids and a molecular mass of 48 kDa, secreted by neurons and neuroendocrine cells, belongs to the granin family [64]. All granins—including CgB and C—are precursors of biologically active substances, involved in a series of biological pathways controlling protein (peptides, hormones, neurotransmitters, and growth factors) secretion upon secretagogue stimulation. CgA-derived peptides include vasostatins [65], pancreastatin [66], and catestatin [67]. Although all granins may be considered as biochemical markers of NENs, as recently reported for vasostatin [68], CgA is the only one routinely used in clinical practice. CgA is synthesized at the rough endoplasmic reticulum, then transported to the Golgi complex, packaged together with other secretory proteins (i.e., hormones and peptides) into immature granules, and then secreted by mature granules by exocytosis [61, 69]. The assessment of circulating CgA levels can be performed by several commercially available kits, which differ in methodology but all rely on antibody-dependent assays such as enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA), radioimmunoassay (RIA), and the more recent immunofluorescent assay based on time-resolved amplified cryptate emission (TRACE). Recently, a further method has been described [70] which employed a non-labeled monoclonal anti-CgA antibody and demonstrated highly sensitive CgA detection. CgA may be assessed in plasma or serum. A significant, positive relationship ($r = 0.9858$, $p < 0.0001$) has been reported between serum and plasma CgA, suggesting that either measurement provides an adequate estimate of circulating CgA [71].

Independently from the method used, CgA is found throughout the diffuse neuroendocrine system and has shown an overall sensitivity of 96% and 75% in functioning and nonfunctioning NENs, respectively, and a specificity ranging from 68% to 100% [72–77]. These diagnostic performances are only estimates of real operative characteristics of CgA, and these estimates often came from heterogeneous, undersized, case-control, uncontrolled studies. Nevertheless, CgA is generally considered a sensitive neuroendocrine marker, whereas its specificity might decrease (up to 68%), as it can be falsely positive in several conditions. CgA can raise in patients with other malignancies such as prostate cancer, small-cell lung cancer, breast cancer, colon-rectal cancer [78, 79], pancreatic adenocarcinoma, and hepatocellular cancer [2, 80] and different settings, including PPI therapy, steroids, and other drugs, chronic atrophic gastritis type A, renal insufficiency, untreated hypertension, liver disease, and inflammatory bowel disease [5, 81] (Table 5.3). Of note, treatment with PPIs induces hypergastrinemia, which in turn results in hyperplasia of enterochromaffin-like neuroendocrine cells; CgA levels can, therefore, increase (up to seven to ten-fold) in patients undergoing therapy with PPIs, and elevated concentrations can be observed up to 2 weeks following treatment discontinuation [82]. Moreover, CgA should always be measured in the fasting state, as food intake is likely to increase CgA levels, therefore, increasing the risk of false positives [83]. Furthermore, CgA levels are not always increased in all the patients with NENs, and normal levels can be found in almost all appendiceal NENs, most insulinomas, many

Table 5.3 Conditions associated with increased levels of Chromogranin A (CgA)

Conditions
Neoplasms Neuroendocrine neoplasms (NENs) Non-neuroendocrine neoplasms (prostate cancer, small-cell lung cancer, breast cancer, colon-rectal cancer, pancreatic adenocarcinoma, hepatocellular carcinoma, ovary cancer)
Diseases of the cardiovascular system Hypertension, heart failure, acute coronary syndrome, giant cells arteritis
Renal diseases Renal insufficiency
Gastrointestinal and liver diseases Chronic autoimmune atrophic gastritis, inflammatory bowel diseases, chronic hepatitis, liver cirrhosis, pancreatitis
Endocrine diseases Pheochromocytoma, hyperparathyroidism, pituitary tumors, medullary thyroid carcinoma, hyperthyroidism
Systemic inflammatory diseases Systemic rheumatoid arthritis, systemic lupus erythematosus, chronic bronchitis
Medications Proton pump inhibitors (PPIs), H-2 blockers intake, steroids

pulmonary NENs, tumors in the duodenum and rectum, some MEN-1 cases as well as poorly differentiated NEN [72, 84]. Caution is therefore suggested in its interpretation. In addition, one should keep in mind that CgA should not be considered a viable tool for screening [85].

While its role in tumor diagnosis is limited by several confounding factors, CgA is currently the most used liquid biomarker in the follow-up of NENs, as its concentration well correlates with disease progression and response to treatment [62, 86], and a correlation between tumor burden and serum CgA has been proven as well. In fact, both advanced tumor stages and the presence of metastases correlate with serum CgA levels [87, 88]; furthermore, a reduction in serum CgA concentrations in subjects undergoing treatment is a suggested surrogate marker of response to therapy. CgA levels decrease in cases of an adequate response, possibly even to the point of normalization, whereas persistently high concentrations are associated with poor clinical prognosis [62, 89, 90]. However, the measurement of CgA is less reliable than advanced imaging techniques, such as magnetic resonance imaging (MRI) or computed tomography (CT), which can also provide the morphological information needed for RECIST criteria (Response Evaluation Criteria In Solid Tumors) [91] and which can, therefore, provide additional information concerning the outcomes of treatment.

According to a recent meta-analysis, CgA seems to be an accurate marker to detect tumor recurrence/progression of GEP-NENs, and CgA levels should be always measured at first diagnosis and repeated during follow-up, particularly in those patients with baseline impaired levels [63].

In summary, CgA seems to be a reliable marker to monitor disease progression and response to treatment and for the early detection of recurrence after treatment, thus being more useful in the follow-up setting rather than in the diagnostic phase [63]. However, further studies are warranted to draw more robust conclusions, including specific cutoff levels to detect tumor recurrence.

5.3.2 Chromogranin B (CgB)

Chromogranin B (CgB) is the second most abundant member of the chromogranin family. Like CgA, it is a strongly acid protein containing approximately 25% acidic amino acid residues. It has 14 dibasic cleavage points but has been less well studied than CgA. Of note, CgB seems not to be affected by renal failure, atrophic gastritis, PPI therapy, and in tumors where CgA is not found (e.g., MEN1 patients and duodenal or rectal NENs), CgB may be increased, which explains the interest to measure CgB in addition to CgA in patients with GEP NENs [4, 23]. However, no robust evidence is available regarding the possible role of CgB as a neuroendocrine marker.

5.3.3 Neuron-Specific Enolase (NSE)

Neuron-specific enolase (NSE) is the neuron-specific isomer of the glycolytic enzyme 2-phospho-D-glycerate hydroxylase or enolase and is found in neurons and neuroendocrine cells.

NSE levels seem not to be related to any secretory activity of the tumor [10, 92]. NSE was introduced as a marker for neuroendocrine cells particularly to be used in the diagnosis of malignant tumors, and it was the first marker used to identify neuroendocrine cells [93]. However, assessment of NSE alone is rarely adequate for diagnostic purposes of NENs, given that only 30 to 50% of them secrete NSE [8, 77, 94]; additionally, NSE has low specificity and sensibility for differentiating NEN from non-endocrine tumors [7]. In fact, patients with other diseases, including thyroid cancer, prostate carcinoma, neuroblastoma, and small-cell lung carcinoma (SCLC), often show elevated levels of NSE [47], whereas patients suffering from neuronal damage exhibit decreased levels of NSE [95]. Of note, whenever a pulmonary mass is present, the detection of increased NSE levels is generally suggestive of an underlying SCLC with a negative prognostic significance. In details, overexpression of NSE by all tumors, including NENs, is usually suggestive of poorly differentiated tumors, and thus of poor prognosis for higher grade cancers [47]. Furthermore, the persistence of increased NSE levels after treatment is usually considered a negative prognostic marker for SCLC, even if the actual significance of post-treatment NSE levels for NENs is far from being clearly understood. In the recent study by Yao et al. [96], data on the impact of biomarkers on overall survival (OS) from the RADIANT-3 study were analyzed and NSE turned back to represent a poor prognostic factor for OS.

In summary, assessing NSE and CgA at the same time as part of the diagnostic process could increase the reliability of their measurement, providing further proof of the presence of an NEN; however, given the nonspecific nature of both markers, these tests provide little information concerning the site of the primary tumor.

5.3.4 Pancreatic Polypeptide (PP)

Pancreatic polypeptide (PP) is a single chain, 36-aminoacid peptide arising from the PP cells of the pancreas and is expressed in neuroendocrine cells of the gut and the pancreas. The function of PP is to self-regulate pancreatic secretion activities (endocrine and exocrine), and it also has effects on hepatic glycogen levels and GI secretions [8, 97]. Before methods for the measurements of CgA were available, PP was used as a general marker for NENs, although it is poorly specific. As a matter of fact, PP has been generally considered a marginal NEN marker with poor utility in everyday clinical practice, due to its low sensitivity and specificity (63% and 81%, respectively) [6]; in fact, less than half of pancreatic NEN patients show elevated serum PP [7]. Furthermore, serum concentrations of PP can be increased in several conditions, such as physical exercise, hypoglycemia, food intake, renal impairment, chronic inflammation, alcoholism, and elder age [8], as well as decreased by somatostatin and hyperglycemia. Moreover, PP has shown to be impaired in acute and chronic pancreatitis, even if determining PP in pancreatitis is quite controversial [98]. It has been hypothesized that the combination of PP with another marker, most commonly CgA, may increase diagnostic capability [99, 100], even if the diagnostic efficacy for the combination of CgA, PP, and gastrin analyzed in the setting of MEN-1 patients was still very low (AUC = 59.6%) [101, 102]. Given that 93% of its secretion can be traced back to the F cells in the pancreas [97], PP has always been considered most likely suggestive for pancreatic NENs; nevertheless the specificity of PP for the pancreatic origin of the primary tumor is not satisfactory as increased serum levels of PP have been reported in other GI NENs as well [7], thus caution is mandatory when interpreting PP level alterations. However, a decline in PP levels after any treatment can be considered as a good prognostic marker.

5.3.5 Human Chorionic Gonadotropin and Alpha-Fetoprotein

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein that is physiologically synthesized during pregnancy by the placenta. As

a protein heterodimer, hCG is composed of two different subunits, named α and β with different characteristics. The α subunit is basically shared with the pituitary hormones such as LH (luteinizing hormone), FSH (follicle-stimulating hormone), and TSH (thyroid-stimulating hormone), whereas the β subunit (β-hCG) is unique. Various endocrine tumors, as well as non-endocrine, exhibit different patterns of expression for hCG [103], as tumors often lack the mechanisms to pair the two subunits. In detail, pituitary tumors and NENs are often characterized by increased expression of α subunit, while the β subunit is often secreted by pancreatic tumors. However, hCG is rarely used in everyday clinical practice for NENs [17].

Alpha-fetoprotein (AFP) is a peptide hormone produced by the yolk sac and the fetal liver during development. In adults, AFP has been historically considered as a biomarker for hepatocellular carcinoma [104] and testicular non-seminomatous germ cell cancer [105]. Increased serum AFP levels have been reported in NENs, suggesting its possible role as a marker for diagnosis [106, 107]; however, more recent evidence suggests that AFP might play a role as a marker of cellular dedifferentiation rather than representing a biomarker per se [108]. The decrease of AFP often highlights an adequate treatment, although the validity of this finding in the context of NENs is still far from being clearly understood.

In testicular tumors, combining hCG with other similar markers, such as AFP, could improve the efficacy of the measurement [108]. However, assessment of hCG and AFP is generally not recommended in NENs, since both lack the sensitivity or specificity of CgA.

5.4 Novel Circulating Markers

Since 1942, at least 40 circulating monoanalytes of different sensitivity and specificity have been developed [72]. The most recent developments have explored the use of new molecular marker technologies: in particular, a great interest has focused on the development of methods to detect circulating tumor cells (CTC), molecular multianalytes (miRNA), and circulating gene transcripts (known as NETest®). However, even if encouraging results have been found so far, these markers are costly and they have not yet been incorporated into routine clinical practice.

5.4.1 Circulating Tumor Cells (CTC)

Circulating tumor cells (CTCs) are released into the bloodstream from both primary tumor and secondary sites of disease and are considered metastatic precursors [109]. CTCs were first detected in patients with NEN in 2011 [110]. Khan and colleagues [111] demonstrated epithelial cell adhesion molecule (EpCAM) expression in NEN by immunohistochemistry. In details, in 79 patients with metastatic NENs, CTCs were detected in the midgut (43%), pancreatic (21%), and bronchopulmonary NENs (31%), and of note, the presence of CTCs had a prognostic significance as it was associated with disease progression, whereas their absence correlated with stable disease. Again, further evidence suggested that CTCs were associated with increased burden, increased tumor grade, elevated CgA, worse progression-free survival, and OS, being an independent prognostic factor for survival [112].

5.4.2 miRNA

The miRNAs are a family of 21- to 25-nucleotide small RNAs that regulate gene expression at the posttranscriptional level by binding to target RNAs, resulting in RNA degradation and inhibition of translation [113]. Several studies have reported the expression of miRNAs in pulmonary carcinoids [114–116], whereas data on GEP-NENs are scarce. However, both pancreatic and small bowel NEN progression appears to be characterized by a differential pattern of miRNA expression, even if with very little or no application of these findings in routine clinical practice so far.

5.4.3 Circulating Gene Transcripts

A multianalyte transcript assay with algorithmic analysis, namely NETest®, has been recently developed for NENs, and its efficacy has been compared with CgA. The NETest ® allows the objective measurement of multiple NEN-related genes in the blood [117]. The test is based on mRNA extraction from ethylenediaminetetraacetic acid (EDTA)-treated blood and subsequent cDNA production measured by polymerase chain reaction (PCR) [118]. Results are expressed as an activity index (NETest score) from 0 to 100 [119]. The normal score cutoff is less than 20%; NETest values between 21% and 40% represent stable disease, while values 41 and 100 reflect progressive disease [120]. The direct analysis of NEN-related genes limits the risk of test alterations due to food, medication, gender, ethnicity, or age [121]. According to available studies, NETtest appears to be more accurate than CgA for both NEN diagnosis [121] and in the follow-up phase [119]. As regard the diagnosis, NETest® accurately correlates with CT/MRI (92%) and functional imaging (94%) [120]. In the follow-up of GEP-NEN patients, the NETest® had demonstrated both prognostic and predictive utility. NETest® is effective in assessing the response of surgical treatment, SSA therapy, and peptide receptor radionuclide therapy. Moreover, the test has been shown to precede radiological progression by 6–24 months, allowing early implementation of effective treatment [119, 120, 122, 123].

However, to date, NETtest® is far more costly and less widely used than CgA in routine clinical practice.

5.5 Conclusion

Numerous biochemical markers have been identified which might be useful in the diagnosis and the follow-up of GEP-NENs; however, only a few are characterized by satisfactory both specificity and sensitivity. Circulating tumor biomarkers can be divided into general and specific biomarkers, the latter characterizing specific clinical syndromes (Table 5.1).

Among generic markers, CgA is the best known, available and used marker. However, it is not highly specific to GEP-NENs as it can be found in other malignancies and other non-tumor–related conditions. According to a recent meta-analysis [63], CgA seems to be more reliable when used to monitor disease progression and response to treatment and for the early detection of recurrence after treatment rather than in the diagnostic setting. It is not useful as a screening test.

Of note, new biomarkers have been developed with the use of new technological molecules: circulating tumor cells, molecular multianalytes (miRNAs), and circulating gene transcripts (NETest®) [72]. According to a recent study, the NETest® seems the most encouraging tool and probably it should be preferred over CgA in both the diagnostic and the follow-up setting due to its better accuracy [124]. However, these new markers are costly and not widely available in everyday clinical practice, and as a matter of fact, CgA is still considered as the most available general biomarker for NENs [6].

The dosage of specific markers is useful for marking the presence of clinical syndrome rather than a tumor. Specific markers include 5-HIAA, insulin, gastrin, VIP, glucagon, somatostatin, and GHRH. Among them, 5-HIAA is an accurate marker for carcinoid syndrome and its accuracy is particularly elevated when its levels are two-fold the upper normal limit. Furthermore, a strong correlation between 5-HIAA circulating levels and carcinoid heart disease onset and progression has been observed, which needs to be taken into account in the clinical evaluation of patients with carcinoid syndrome.

In summary, circulating biomarkers both general and specific offer a useful diagnostic tool in conjunction with radiology and tissue pathology for NENs. It is important to keep in mind that biomarkers both general and specific should be measured when there is a strong suspicion of NEN and never as a screening tool due to the high numbers of false-positive results; moreover, they are still widely used in the clinical practice, although caution is necessary when interpreting their results due to the high number of confounding factors that might affect their accu-

racy. They are more reliable when used in the follow-up of GEP-NEN patients rather than in the diagnostic setting. Biomarkers of diagnostic and prognostic value for NENs are urgently needed to improve patient management and tailor the therapeutic approach for each patient.

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