Neuroendocrine Tumors Biomarkers

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Marialuisa Appetecchia, Rosa Lauretta, Francesca Rota, and Massimo Carlini

The diagnosis of neuroendocrine tumors (NETs) is challenging due to the non-specificity of the symptoms. Since NETs produce and secrete a large variety of bioactive substances, it is important to identify those that can be used in clinical practice as biomarkers with a diagnostic, prognostic or predictive role. An ideal diagnostic biomarker should have high sensitivity and specificity, as well as the ability to discriminate the tumor site and the stage of disease; in addition, it should have prognostic significance and be able to be used in the follow-up to evaluate the effectiveness of therapy and the progression or relapse of the disease.

Neuroendocrine markers are divided into *non-specific*, present in all NETs, and *specific*.

5.1 Non-Specific Biomarkers

The most important non-specific NET marker is chromogranin A (CgA). CgA is part of the granin family, which includes eight proteins: CgA, CgB or secretogranin I (SgI), CgC or secretogranin II (SgII), SgIII, SgIV, SgV or 7B2, SgVI or NESP55 (neuroendocrine secretory peptide), and VGF [1–4]. These proteins are the principal components of dense-core secretory vesicles in neuroendocrine cells, probably play an important role in regulating the function of secretory granules and are precursors of biologically active peptides. In clinical practice only CgA is used, an acidic protein of 439 amino acids with a molecular mass of 48 kDa. CgA contains a disulfide bridge in the N-terminal region, which is used for some CgA-related activities, and 10 pairs of basic amino acids, which, along with other sites in the molecule, can be subjected to cleavage by endogenous

M. Appetecchia (✉)
Endocrinology Unit, Regina Elena National Cancer Institute
Rome, Italy
e-mail: marialuisa.appetecchia@ifo.gov.it

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proteases. Several biologically active peptides derive from proteolysis of CgA, such as pancreastatin, catestatin and vasostatins I and II. CgA and CgA-related peptide perform many biological activities. In fact, even though their role is not definitively clarified, they act on endocrine organs such as parathyroid glands, adrenal glands and endocrine pancreas, and on the cardiovascular system and adipose tissue.

CgA is a pan-neuroendocrine marker and it is the best available biomarker for NETs. However, several problems are related to its use in clinical practice and so it is far from the ideal marker. CgA levels are abnormal when they exceed by two-threefold the upper normal range. As CgA levels increase after food intake, they should be tested in fasting patients [2]. The determination of CgA may be done on plasma or serum, since a strong positive linear relationship has been reported between both types of tests ($r = 0.9858$, $P < 0.0001$) [5]. There are several commercial assays for the measurement of circulating CgA concentrations. Stridsberg et al. compared three methods of assay of CgA: CgA RIA-CT (CIS Bio International, Gif-sur-Yvette Cedex, France), Dako CgA ELISA kit (Dako A/S, Glostrup, Denmark) and CgA EuroDiagnostica (ED) (Malmö, Sweden). CgA was measured with the three methods in 77 patients. Forty-six patients had NETs, 31 patients were considered not to have NET or to be tumor-free after radical surgery.

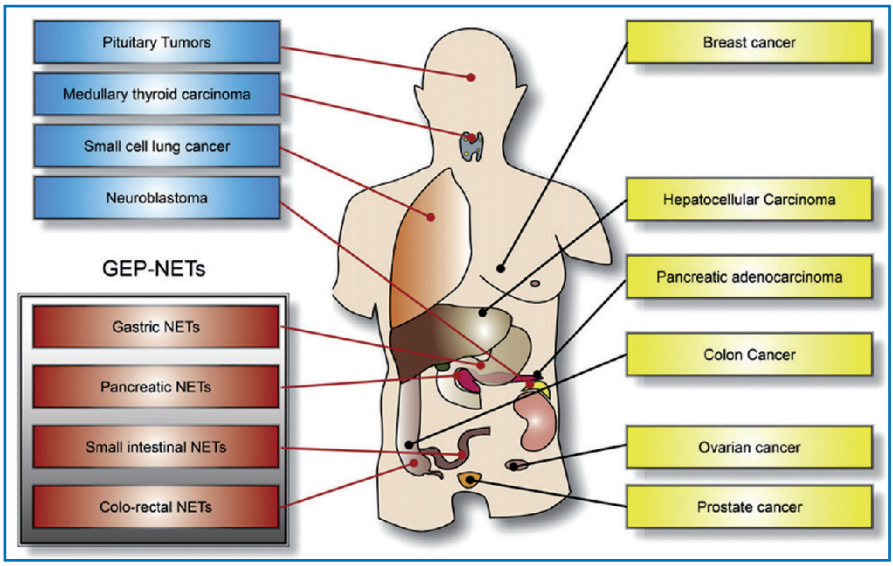

Fig. 5.1 Neoplastic causes of elevated Chromogranin A (CgA). CgA elevations occur in different types of NETs but are usually more pronounced in GEP-NETs (small intestinal, gastric, and pancreatic NETs). CgA elevations may occur in carcinomas with a complete or a partial neuroendocrine phenotype (left and right box stacks, respectively). In HCC, the cause of CgA elevation may reflect impaired metabolism of CgA fragments due to concurrent liver failure (Reproduced with permission from [7])

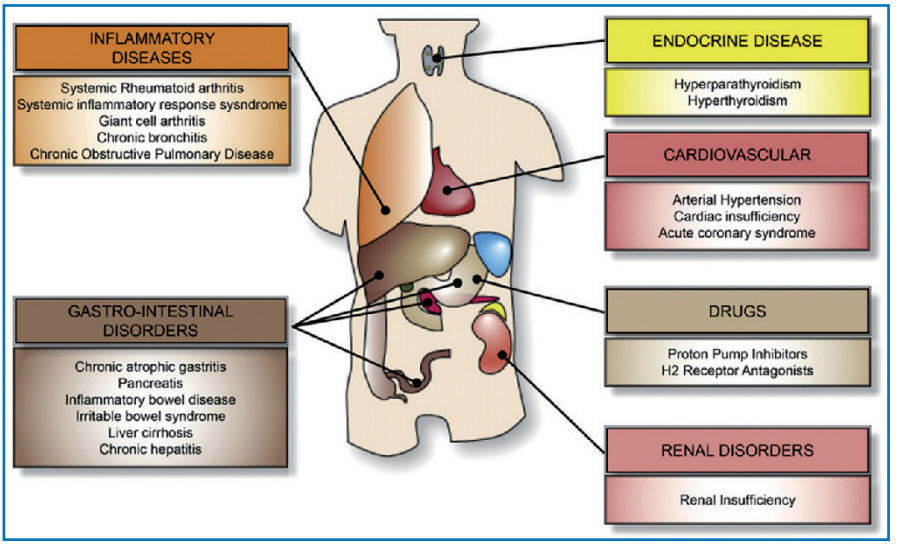

Fig. 5.2 Non-neoplastic causes of Chromogranin A (CgA) elevation. CgA is elevated in endocrine diseases, chronic and acute inflammation, and cardiac insufficiency. Acid-suppressive medications result in hypergastrinemia (G cell and ECL cell hyperplasia) and a concomitant increase in cosecreted CgA. Renal failure increases detectable plasma CgA (p-CgA) by reducing glomerular filtration of CgA-related peptides. P-CgA alone cannot discriminate between GEP-NETs, pancreatitis, inflammatory bowel disease, irritable bowel syndrome, or hepatitis (Reproduced with permission from [7])

The results obtained with different methods are not comparable so, in the same patient, CgA should always be measured always with the same method [6]. Although, among all markers, CgA presents the best combination of sensitivity and specificity, there are numerous cases of false negatives and especially false positives. In fact CgA levels increase in several neoplastic and non-neoplastic conditions [2, 7] (Figs. 5.1 and 5.2). Some non-neuroendocrine carcinomas, such as prostate cancer, small-cell lung cancer, breast cancer, colorectal cancer, may have a neuroendocrine differentiation and be associated with increased levels of CgA [8–11]. Higher CgA values were even detected in pancreatic adenocarcinoma and hepatocellular cancer, but the pathophysiological meaning is unknown [2, 7, 12]. Increased CgA levels were also found in endocrine disease such as pheochromocytoma, hyperthyroidism, hyperparathyroidism, pituitary tumors, medullary thyroid carcinoma, and in non-neoplastic conditions, i.e., gastrointestinal disorders, cardiovascular and inflammatory diseases [2, 7]. However, the most common causes of false positive results in clinical practice are renal failure and drugs. Decreased CgA clearance can increase circulating CgA levels in proportion to the degree of renal failure, up to values similar to those found in NETs.

Proton pump inhibitors (PPIs) and, to a lesser extent, H2-receptor antagonists, are a frequent cause of CgA elevation. PPIs inhibit gastric acid secretion, enhancing gastrin release by the antral G cells. The resultant hypergastrinemia

causes hyperplasia of enterochromaffin-like neuroendocrine cells with consequent increase in CgA. CgA levels increase more than tenfold with PPIs use. The PPI effects are early, presenting within the first six days after the start of therapy, and persist for 1–2 weeks after discontinuation of the drug [13].

CgA, however, lacks specificity and is not to be used for population screening in the absence of strong clinical or radiological evidence of tumor presence [14]. The guidelines recommend CgA serum determination for the diagnosis and follow-up of all NETs [15–19]. A recent meta-analysis demonstrated that circulating CgA was an efficient biomarker for the diagnosis of NETs with high sensitivity and specificity (73% and 95%, respectively) [20]. Many studies investigated the diagnostic accuracy of CgA compared to other general biomarkers. Bajetta et al. studied the role of CgA, neuron-specific enolase (NSE), carcinoembryonic antigen (CEA), and urinary 5-hydroxyindole-3-acetic acid (5-HIAA) in 127 patients with NETs, including gastroenteropancreatic NETs (GEP-NETs). CgA was the best marker (a specificity of 85.7% and sensitivity of 67.9%) compared to NSE, CEA and 5-HIAA. CEA had a sensitivity of only 15.4%; NSE and 5-HIAA showed a very high specificity (100%) but a lower sensitivity (32.9% and 35.1%, respectively) [21].

Other studies demonstrated a better diagnostic accuracy of CgA than NSE, 5-HIAA, α-subunit of glycoprotein hormones and pancreatic polypeptide. There is scientific evidence that the simultaneous detection of CgA and pancreatic polypeptide significantly improves the sensitivity of CgA (96% vs. 84%) in patients with GEP-NETs [22]. Some studies suggested an association between type of NET and increased CgA levels. The highest values were found in ileal NET and GEP-NET associated with MEN1, whereas intermediate values were detected in functioning and non-functioning pancreatic NET, type II and III gastric NET and Zollinger-Ellison syndrome in MEN1. Type I gastric NET, pituitary, and parathyroid tumors have lower levels [2].

The diagnostic accuracy of CgA measurement also varies with the degree of differentiation, being more frequently elevated in well-differentiated tumors than in poorly differentiated ones [2]. This may be due to the greater functional integrity of the secretory system in more differentiated neuroendocrine cells. In a study on 63 NET patients including 35 patients with GEP-NETs, the sensitivity of CgA levels for detecting well-differentiated carcinoids, well-differentiated neuroendocrine carcinomas and poorly differentiated neuroendocrine carcinomas was 58%, 68% and 37%, respectively. The specificity was 100% for patients with well-differentiated carcinoids and neuroendocrine carcinomas, but only 67% for well-differentiated neuroendocrine carcinomas [23].

In well-differentiated NETs, there was indirect evidence for a prognostic role of CgA. In fact, the CgA levels were related to tumor stage and advanced stages were associated with reduced survival. CgA concentrations were higher in patients with extensive metastases than in those with localized disease or with limited hepatic metastases [2]. Gastrinomas may be an exception because they are associated with high circulating CgA levels even in the absence of hepatic

involvement [24]. In GEP-NETs the diagnostic accuracy of CgA is higher in functioning versus non-functioning tumors [25], as well as in metastatic versus loco-regional NETs and in well-differentiated versus poorly differentiated tumors [2].

It has been suggested that, due to the CgA-related tumor burden, a change in CgA concentrations may indicate a response to treatment. To assess whether CgA is a predictive marker of response to treatment with somatostatin analogs (SSAs), the octreotide test was developed. CgA levels are measured 0, 3 and 6 hours after the i.v. injection of 200 mcg octreotide. Massironi et al. evaluated whether plasma CgA levels in response to the octreotide test predicted the clinical response to SSAs in GEP-NET patients. They concluded that a decrease of CgA greater than 30% after the octreotide test identifies the patients most likely to be responsive to SSA therapy [26]. It should be noted that SSAs reduce CgA as a result of its effect not only on tumor burden, but also on the secretory activity of neuroendocrine cells [27]. Therefore, the reduction of disease burden can be evaluated through CgA only if the dose of SSAs does not vary over time [28]. In pancreatic NET patients treated with everolimus in the RADIANT-1 study, increased baseline CgA and NSE circulating levels were predictive markers of survival and progression-free survival [29, 30]. Jensen et al. showed that a reduction in CgA circulating levels greater than or equal to 80% following cytoreductive surgery for a carcinoid tumor with hepatic metastases was predictive of subsequent complete symptom resolution and disease control. Substantial reduction in CgA was associated with improved patient outcomes, even after incomplete cytoreduction [31]. Recently, it was confirmed that CgA could have a role in identifying disease recurrence. In 152 patients with jejunal, ileal and pancreatic NETs, CgA proved to be a predictor of disease recurrence 6 months before radiological progression, according to RECIST 1.1 criteria [32]. Massironi et al. reported increased CgA levels 9–12 months prior to clinical and radiological relapse in 15 GEP-NET patients who recurred after radical surgery [25].

Another unspecific marker used in NETs is NSE, which is the neuron-specific isomer of the glycolytic enzyme 2-phospho-D-glycerate hydrolyase or enolase. NSE is found in neurons and neuroendocrine cells. Circulating NSE levels have been reported to be increased in patients with thyroid cancer, prostate carcinoma, neuroblastoma, small cell lung carcinoma (SCLC), and pheochromocytoma. NSE has a very good sensitivity in SCLC and a good discriminatory power between SCLC and non-small cell lung cancer [33]. NSE levels are increased in 30-50% of NET patients, particularly in those with a poorly differentiated tumor [1, 34, 35]. However, because of its low diagnostic accuracy, NSE is inadequate for diagnostic and prognostic use [36–38].

Pancreatic polypeptide (PP), secreted by the PP cells of the pancreatic islet cells, is a marker with low diagnostic accuracy (63%sensitivity and 81%specificity) [39], but in the diagnosis of GEP-NETs, a combined assessment of PP and CgA leads to a significant increase in sensitivity, particularly in non-functioning pancreatic NETs [22].

Other unspecific markers, such as alpha and beta subunits of human chorionic gonadotropin (hCG) have a limited clinical usefulness [40].

A recent consensus agreed that current general blood biomarkers, including CgA, are inadequate [36-38], and new biomarkers have been proposed, including circulating tumor cell and multianalyte biomarkers, such as microRNA and mRNA [36–38, 41–43]. An mRNA-based, specific multianalyte assay with algorithmic analyses has been shown to have better sensitivity and specificity than CgA in initial clinical studies. [42, 43].

5.2 Specific Biomarkers

The specific markers are typical of the functioning NETs and vary according to the tumor hormone production, which causes a specific clinical syndrome.

5.2.1 Serotonin

Serotonin (5-HT) is a biogenic amine derived from tryptophan. It is stored and secreted by enterochromaffin cells of the gastrointestinal tract (80%), platelets (storage only) and serotoninergic neurons of the central nervous system. 5-HT is a potent vasoconstrictor and acts as a regulator of gastrointestinal motility, mood, appetite and sleep. The urinary metabolite of serotonin is 5-hydroxyindole acetic acid (5-HIAA) which is particularly useful in the diagnosis and follow-up of patients with carcinoid syndrome [44]. High-performance liquid chromatography (HPLC) is the currently recommended assay for the measurement of urinary 5-HIAA.

As the determination of this metabolite is sensitive, the sample should be kept away from direct light and refrigerated [28]. Written instructions should

Table 5.1 Confounding agents of urinary 5-HIAA levels

Increase 5-HIAA levels		Decrease 5-HIAA levels
Medication	**Food**	**Medication**
Acetaminophen	Avocado	Aspirin
Antihypertensive	Banana	Ethyl alcohol
Caffeine	Eggplant	Heparin
Diazepam	Pineapple	Imipramine
Ephedrine	Plantain	Isoniazid
Glyceryl guaiacolate	Plum	Levodopa
Nicotine	Tomato	MAO inhibitors
Phenobarbital	Walnut	Methyldopa, tricyclic antidepressants

From: Albertelli M, Campana D, Pelosi G (2016) Marker tumorali. Select 2, 2016 (reprinted with permission of Thenewway Srl).

be handed out to patients including food and medications that could falsely increase urinary 5-HIAA levels [45]. A diet free of these confounding agents should be carried out within three days before the urine collection [45–47] (Table 5.1). Certain co-morbidities or associated disorders may have effects on the concentration of 5-HIAA. Falsely low 5-HIAA levels may be encountered in patients with renal impairment and those on hemodialysis. 5-HIAA may increase in untreated patients with malabsorption [48–50].

Overall sensitivity and specificity of urinary 5-HIAA is 70% and 90%, respectively [48]. Monitoring levels of 5-HIAA allows checking of the secretory activity of carcinoid tumors and serves as an objective marker of biochemical response to treatment with antisecretory agents such as somatostatin analogs [1]. As an intra-individual variability in 5-HIAA values exists, especially in the diagnostic phase, it is recommended to carry out the examination twice so as to obtain an average value of 5-HIAA [28].

5.2.2 Gastrin

Gastrin is a peptide hormone that stimulates secretion of gastric acid (HCl) by the parietal cells of the stomach and acts in gastric motility. It is released by G cells in the pyloric antrum of the stomach, in the duodenum and pancreas. Zollinger-Ellison syndrome (ZES) is caused by gastrin producing tumors (gastrinomas) and is characterized by recurrent peptic ulcers and secretory diarrhea. The diagnostic marker of this condition is fasting serum gastrin (FSG), which is usually elevated (more than tenfold the upper limit of normal (ULN) in the presence of a low gastric pH [24, 51, 53, 56, 57]. FSG alone is not adequate to make the diagnosis of ZES because hypergastrinemia may be present in achlorhydric patients with chronic atrophic fundus gastritis and in other conditions associated with hyperchlorhydria (i.e., *Helicobacter pylori* infection, gastric outlet obstruction, renal failure, antral G-cell syndromes, short bowel syndrome, retained antrum) [51–53].

The chronic use of proton pump inhibitors (PPIs) [52–55] leads to high FSG levels and a gastrin provocative test is needed to establish the diagnosis of ZES [24, 51, 52, 56, 57] as well as the assessment of the gastric pH [58].

The current recommended criterion for the diagnosis of ZES depends on FSG elevation (24, 51, 53, 58). In the presence of hypergastrinemia (FSG: two- to tenfold the ULN or greater than tenfold the ULN) with gastric pH less than 2, a complete gastric analysis is recommended prior to performing a secretin test [24, 51, 58, 59].

The patient must undergo esophagogastroduodenoscopy (EGDS) with gastric antral and fundus biopsies ± a serum test for antiparietal and intrinsic factor antibodies to exclude an atrophic fundus gastritis, *Helicobacter pylori* testing and 24-hour pH-metry (basal acid output pre and post secretin is recommended) [28].

After establishing that the patient has no active peptic disease, PPIs should be interrupted 10 days to 2 weeks prior to the test and replaced with high doses of H2 blockers (ranitidine 600 mg every 4–6 hours) for 5–7 days. Ranitidine

should then be stopped and a liberal use of antacids can be started. The secretin test should be done in 12–24 hours [53, 59]. However, the interruption of all antisecretory medications should be individually adapted. Patients should be warned about the acute exacerbation of symptoms. In these cases, antisecretory drugs can be restored.

The secretin test should be performed with the patient fasting (12–14 h). Secretin (2 U/kg body weight) is given by i.v. bolus and gastrin serum is measured at baseline, at −15 and −1 min before the test and at 2, 5, 10, 15, 20 and 30 min after secretin. Possible side effects of the secretin test include flushing and allergic reaction. Blood samples must be collected in heparinized vacutainers and placed in ice. An increase in circulating gastrin levels compared to baseline data (delta) of at least 200 pg/mL at any time during the test is considered diagnostic of autonomous secretion [56]. The National Institutes of Health (NIH) reduced the gastrin delta to ≥120 pg/mL with a sensitivity of 94% and specificity of 100% [24]. If the secretin test is negative but the suspicion of ZES remains high, a calcium stimulation test may be helpful, as it may be positive in 5–10% of such cases [60, 61]. Calcium gluconate 255 mg/3 mL is injected intravenously in 30 seconds. As done in the secretin test, venous blood sampling is performed before and at 2-min intervals up to 10 min after calcium injection [61]. When diagnostic, serum gastrin gradients show increased values greater than 20% above baseline at any time point (2, 4, or 6 min after i.v. calcium injection) usually with gastrin values higher than 300 pg/mL [61].

An alternative diagnostic test is the glucagon stimulation test, during which glucagon is infused at 20 μg/kg/hours in 30 min [62]. A gastrinoma is suggested when the percentage increase over the basal value of circulating gastrin reaches the peak within 10 min after glucagon administration, with circulating gastrin levels greater than 200 pg/mL [62]. The diagnosis of ZES is supported by the monitoring of the basal acid output (BAO). When BAO is greater than 15 mmol/hours, it is highly suggestive for this diagnosis [28].

5.2.3 Insulin

Proinsulin is the biosynthetic precursor of insulin that is synthesized in pancreatic islet cells. Proinsulin derives from a preproinsulin that acts as a signal for its transport to the Golgi apparatus, where it reaches the correct conformation. Insulin consists of two polypeptide chains linked by disulfide bridges. It is produced by the proteolytic cleavage of proinsulin through a connecting peptide of 33 amino acids. This peptide is called C-peptide, while the enzyme responsible for the proteolytic cleavage is an endopeptidase.

Insulin regulates the metabolism of carbohydrates, fats and protein by promoting the absorption of glucose from the blood into fat tissue, liver and skeletal muscle cells. Excessive insulin secretion leading to hypoglycemia usually results in neurologic and autonomic symptoms.

The diagnosis of insulinoma is suggested in the presence of symptoms of hypoglycemia with glucose values lower than 2.2 mmol/L (40 mg/dL) and relief of symptoms with the administration of glucose [63]. Fasting allows one to check an autonomous insulin secretion because it causes the lowering of glycemia, and in this circumstance insulin secretion should be suppressed. The 72-hour fast test is the gold standard for a biochemical diagnosis of insulinoma [64–66].

Factitious hypoglycemia, due to exogenous insulin, should be suspected in the presence of high (often very high) insulin serum levels, in combination with a suppressed C-peptide. Intake of sulphonylureas and related insulin secretagogues may be diagnosed by a urinary drug test [67]. For the 72-hour fast test the patient should be monitored in a secure inpatient setting. Blood samples for insulin, glucose and C-peptide assay should be obtained at least 2–4 times per day, even when the patient becomes symptomatic. β hydroxybutyrate (or urinary ketones), a metabolite of the oxidation of fatty acids is produced during fasting. It should be measured at the end of the test in order to confirm the validity of the fasting. Symptoms appear within 12 hours in one-third of patients, within 24 hours in 80%, within 48 hours in 90% and within 72 hours approaching 100% [64]. The endpoint of the test is a documented hypoglycemia with blood levels equal to or less than 2.2 mmol/L and concomitant insulin levels greater than 6 μU/L and β hydroxybutyrate levels equal to or less than 2.7 mmol/L. If the results are still equivocal, a glucagon stimulation test after the 72-hour fast test is suggested. Patients with insulinoma respond to glucagon administration (1 mg i.v. push) with a rise in blood serum glucose levels, indicating adequate glycogen stores [68].

Occasionally, insulinoma patients have been reported to exhibit postprandial hypoglycemic symptoms rather than in the fasting state. In such cases, hypoglycemia may be erroneously considered reactive. When performed, the oral glucose tolerance test may provide misleading results, since insulinoma cells may retain their glucose reactivity [69].

5.2.4 Glucagon

Glucagon is a peptide hormone produced by alpha cells of the pancreas. Its effect is opposite to that of insulin as it raises the concentration of glucose in the bloodstream. The diagnosis of functioning NETs producing glucagon (glucagonomas) is established by glucagon circulating levels generally greater than 500 pg/mL (normal <120 pg/mL). However, other diseases can cause hyperglucagonemia, including cirrhosis, pancreatitis, diabetes mellitus, prolonged fasting, sepsis, burns, renal failure, acromegaly and familial hyperglucagonemia [55].

In the majority of cases the diagnosis is done by the finding of hyperglucagonemia combined with clinical symptoms and signs often in the presence of hepatic metastatic disease with a large pancreatic mass, both of which are usually positive at whole-body somatostatin receptor scintigraphy.

5.2.5 Somatostatin (SS)

Somatostatin (SS) is a polypeptide hormone produced by the hypothalamus, the pancreatic delta cells, the gastric antral D cells and the APUD cells. It inhibits the release of pancreatic insulin and glucagon hormones and exocrine enzymes. In addition, it inhibits gastric hydrochloric acid secretion. It also acts as a neurotransmitter and inhibits the secretion of some pituitary hormones (GH, TSH, PRL).

Plasma SS levels are assessed in the suspicion of a SS-secreting tumor (SSoma) which is characterized by glucose intolerance, cholelithiasis and steatorrhea [70]. As there is no specific reagent for the determination of circulating SS levels, SS-like immunoreactivity (SLI) and SS-28, the main molecular form of circulating SLI, are assessed. Other conditions associated with elevated plasma SS levels are medullary thyroid cancer, lung cancer, pheochromocytoma and paraganglioma [5, 71].

5.2.6 Vasoactive Intestinal Peptide (VIP)

Vasoactive intestinal peptide (VIP) is a peptide hormone part of the glucagon/secretin superfamily. VIP is produced in many tissues such as gut, pancreas, and suprachiasmatic nuclei of the hypothalamus. The diagnosis of a VIP-secreting tumor (VIPoma) is suspected in patients with the Verner-Morrison syndrome characterized by severe watery diarrhea, documenting the presence of large volume secretory diarrhea (usually greater than 3 liters per day, with no osmolar gap in the stool fluid) accompanied by dehydration and electrolyte disorders with an increase plasma VIP level, usually greater than 500 pg/mL (normal, less than 190 pg/mL) [55, 71, 72].

Other diseases that can give large volume diarrhea and can mimic VIPomas, but are not associated with increased plasma VIP levels, include Zollinger-Ellison syndrome, chronic laxative abuse, sprue, AIDS, and rarely secretory diarrhea of unknown origin [4, 55, 71].

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